UNIVERSITY OF NAPLES "FEDERICO II"

DEPARTMENT OF AGRICULTURAL SCIENCES

AND

AN-NAJAH NATIONAL UNIVERSITY

FACULTY OF GRADUATE STUDIES

MASTER DEGREES IN

FOOD SCIENCE AND TECHNOLOGY

AND

NUTRITION AND FOOD TECHNOLOGY

Experimental Thesis

EFFECT OF METABOLIC REDUCTION OF PROBIOTICS ON THE FUNCTIONALITY OF FRUIT JUICE

Tutor: Prof. Mauriello Gianluigi **Candidate**: Shaima' Nasser

Co-Tutor: Dr. Mohammad Altamimmi **Matr**. N06001028

Academic year 2020-2021

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Dedication

To my beloved Father and mother, who raised me to be I am today, who have been my source of inspiration and gave me strength when I thought of giving up…

To my beloved brothers and sister who shared their words of encouragement and advice to finish this thesis…

And Lastly, to my teachers who support and encourage me a lot…

I wholeheartedly dedicate this work

Acknowledgment

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In addition, the deepest appreciation to my family who stand beside me and enlightened my academic path with support and care.

الاقرار

أنا الموقع أدناه، مقدّم الرسالة التي تحمل العنوان:

EFFECT OF METABOLIC REDUCTION OF PROBIOTICS ON THE FUNCTIONALITY OF FRUIT JUICE

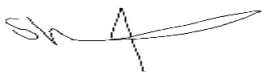
أقر بأن ما اشتملت عليه هذه الأطروحة إنما هو نتاج جهدي الخاص، باستثناء ما تمت الاشارة إليه حيثما ورد. وأن هذه الرسالة كاملة، أو اي جزء منها لم يقدم من قبل لنيل اي درجة او لقب علمي او بحثي لدى أي مؤسسة تعليمية أو بحثية أخرى.

Declaration

The work provided in this thesis, unless otherwise referenced, is the researcher's own work, and has not been submitted elsewhere for any other degree or qualification.

Student's Name: **اسم الطالب: شيماء برهان عبدالرحيم ناصر**

Signature:



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Date: **التاريخ:2021/07/28**

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EFFECT OF METABOLIC REDUCTION OF PROBIOTICS ON THE FUNCTIONALITY OF FRUIT JUICE

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Abstract

Ultrasound (US) and microencapsulation can be regarded as the tools to modulate the metabolism of probiotic bacteria and, consequently, these strategies can be used to reduce the acidification of the probiotic bio-active beverages. Attenuation can be done through the combination of ultrasound (US), and microencapsulation as a physical approach. Furthermore, microencapsulation, through vibrating technology, is also aimed at incorporating probiotic strains in functional foods such as capsules with controlled release of these active strains. About attenuation, there is no data available on the effect of this treatment on the overall profile of probiotic bacteria. So, the major topics of this study: i) to study the effect of ultrasound and microencapsulation tools on the survival, acidification and growth of *Limosilactobacillus reuteri* DSM 17938 (*L. reuteri*) inoculated in tomato juice during its storage for 28 days at 4 and 20 °C, ii) to study the effect of US on *L. reuteri* survival at pH 2.5 and with 0.15% bile salt, iii) to get a product with chemical-physical and sensory characteristics that are acceptable to the consumers. A preliminary screening on sonication was done by using 3 power levels (57, 64 and 78 W) and two different duration time (4 and 6) min. The best combination was 57 W- 6 min; this decision

come from the acceptable post-acidification and viability results after sonication. The effect of this treatment on the functional properties also studied. At low pH 2.5 *L. reuteri* DSM 17938 has a higher survival than the sonicated one. In contrast, the reduction of viability in case incubation in deionized sterile water containing 0.15% of bile salts of non-treated *L. reuteri* DSM 17938 was higher than half sonicated microorganism. The probiotication of tomato juice from market was done in three different ways (with a cell suspension, with a sonicated cell suspension and with microcapsules of the sonicated cell suspension). Post acidification and viability of *L. reuteri* were monitored at 4 and 20 °C for 28 days. This study confirms that the best storage temperature for *L. reuteri* sonicated and microencapsulated (LR-US-MC) tomato juice is 4 °C; the pH (4.13) and viability (7.24 log CFU/ml) value are higher at 4 °C storage temperature than pH (3.87) and viability (7.08 log CFU/ml) value at 20 °C.

Chapter One
Introduction

1.1 Research overview

Health is largely connected to balanced nutrition. Several studies have shown the role of some nutrients in the reduction of the risk of certain diseases such as non-communicable disease (NCDs) that include cardiovascular disease (CVD), type 2 diabetes (T2D), cancer and many others. Therefore, through correct and balanced food choices it is possible to improve the quality of life and prolong it (1) (2). For these reasons the development of new functional food increased and the innovation in food area leads to the creation of new market niches, mainly related to functional products. Japan was the first country that introduced the concept of functional food during the 1980s, it is considered as a source of physical and mental well-being, not only as a source of energy for living (3) (4). The functionality of functional products depends on the bioactive ingredients often included naturally in the food, but a food can be also made functional by adding bioactive components(5). Probiotic foods, therefore, fall into the macro-category of functional foods.

Probiotics are defined as live microorganisms, which exhibit a beneficial effect on the host health after ingestion when taken in adequate amount, due to the improvement of the properties of the native microbiota. Most lactic acid bacteria are considered as probiotics (6).

As probiotics remain alive in the food matrices some sensory attributions are affected. Fruit juices supplemented with probiotics decline in their sensorial quality (7). Slowing the metabolic activity of probiotics using several techniques for attenuation can be hypothesized to maintain the sensory quality during the shelf-life (8). Many indicators related to the metabolic activity of probiotics such as pH, acidity and the presence of its metabolites can be monitored (9) (10) (11).

1.2 Probiotics

The term probiotic derives from the Greek "*pro*" and "*bios*" and literally means "in favor of life". The definition of probiotics has been revisited over the years. Food and Agriculture Organization of the United Nations (FAO) with World Health Organization (WHO), was clarify the definition in 2014 to be as "*Live microorganisms which when administrated in adequate amounts confer a health benefit on the human host*". This definition has been kept mostly stable during the last 17 years. In recent decades, interest in the use of probiotic cultures has seen a significant increase. In fact, the scientific papers about probiotics have grown from 2001 (760 papers) to 2019 (20315) substantially (12) (13).

The diagram below showed the history of probiotics (Figure 1).

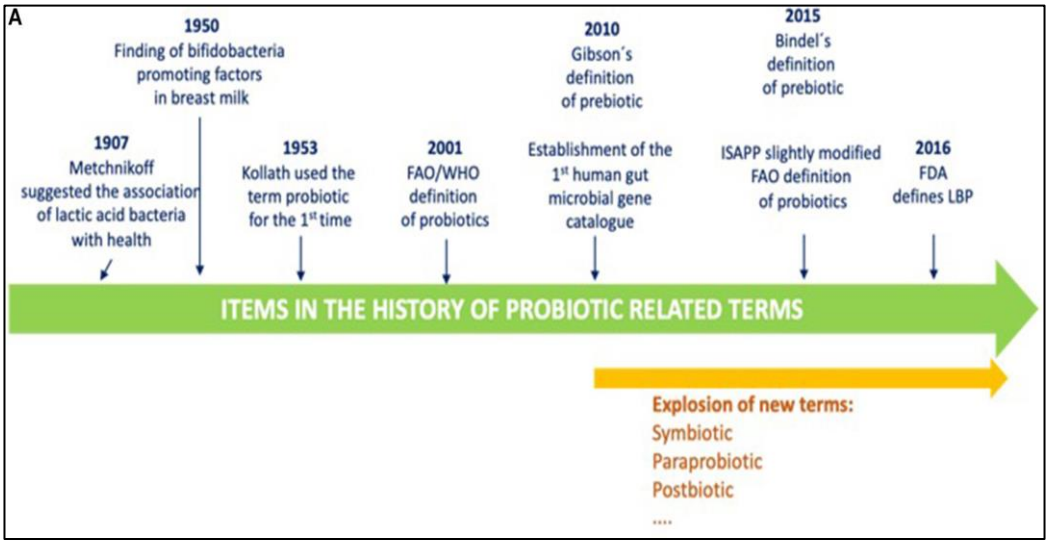

Figure 1: Items selected in the history of probiotics (14).

They have been classified as Generally Recognized as Safe (GRAS). This group includes, basically, *Bifidobacterium* spp. and *Lactobacillus* spp, yeast and other species (Figure 1) (15) (16).

In recent decades, interest in the use of probiotic cultures has seen a significant increase. This trend is the result of the acquisition of new knowledge about the correlation between human health and the gastrointestinal microbiota. The gastrointestinal microbiota carries out important metabolic, immunological and protective functions. Probiotic cultures fortification is connected to many health benefits such as reduction of obesity, reduction of atopic dermatitis, suppression of *Helicobacter pylori* infections, improvement on the symptoms of irritable bowel syndrome, antimutagenic, anticarcinogenic, antidiarrheal properties, stimulation of the immune system, reduction in serum cholesterol, improvement in lactose metabolism, antimicrobial activity, reduction in gastrointestinal infections (Figure 2) (16) (17) (18).

The intestinal microbial ecosystem is composed, at the phyla level, by four main ones: *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria*. The percentages of *Firmicutes* to *Bacteroidetes* (F/B ratio) differ between healthy and unhealthy individuals (19). Besides, the destruction of the normal balance between the host and the microbiota, generally known as dysbiosis, it plays important role in several diseases and syndrome. Probiotics can be used to restore balance of the intestinal ecosystem. Interesting, there is a new trend to use commensal bacteria as probiotics to restore a healthy situation. This leads to the identification of new type of probiotics called Live Bio-Therapeutic Products (LBPs) or Next-Generation Probiotics (NGPs) (15) (20).

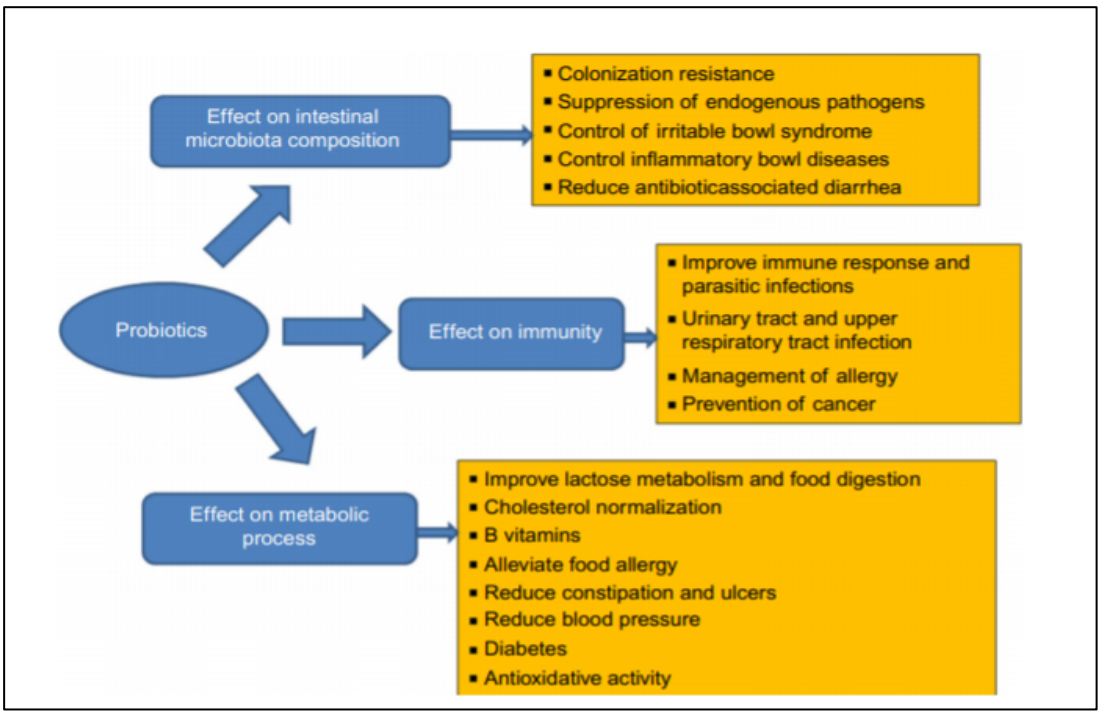

Figure 2: Major health benefits bestowed by probiotic microorganisms (16).

Table 1: List of Microorganisms Used as Probiotics (16)

Lactobacillus Species	***Bifidobacterium Species***	***Yeast and Other Species***
L. acidophilus	*B. bifidum*	*Saccharomyces boulardii*
L. casei Shirota	*B. breve*	*Saccharomyces cerevisiae*
L.delbrueckii spp. Bulgaricus	*B. infantis*	*Streptococcus thermophilus*
L. johnsonii	*B. longum*	*Enterococcus faecalis*
L. reuteri *	*B. adolescentis*	*Enterococcus faecium*
L. rhamnosus	*B. animalis*	*Pediococcus acidilactici*
L. gallinarum	*B. lactis*	*Lactococcus lactis*
L. plantarum		*Leuconostoc mesenteroides*
L. salivarius		*Bacillus cereus*
L. crispatus		*Escherichia coli Nissle 1917*
L. gasseri		*Propionibacterium freudenreichii*

1.2.1 Limosilactobacillus reuteri

Lactic acid bacteria (LAB, Lactic Acid Bacteria) are Gram-positive bacteria with rod, cocci or cocci-bacillary morphology, catalase negative, non-spore-forming, cytochrome-free, air-tolerant anaerobes, nutritionally demanding, acid-tolerant, with strictly metabolism fermentative (21).These are ubiquitous microorganisms that prefer nutrient-rich habitats and are part of the normal microflora of the oral cavity, intestines and human vagina. Its pro-technological role is recognized thanks to its acidifying, proteolytic, lipolytic, flavoring, antioxidant activity. They are used in the production of starter and flavoring cultures, as antimicrobial agents and for the production of probiotic products.

There are several genera attributable to the group of lactic bacteria. Of considerable importance is the genus Lactobacillus, both for its use in the production of fermented foods (dairy products, meat, vegetables and bakery products) and for belonging to the genus of many species to which

probiotic strains are ascribed, such as *Lb. acidophilus*, *Lb. casei*, *Lb. johnsonii*, *Lb. reuteri*, *Lb. rhannosus*, *Lb. salivarius*, *Lb. crispatus* and *Lb. plantarum*. It should be emphasized that the genus Lactobacillus has recently been revised from the taxonomic point of view with the proposal of 23 new genera from species belonging to this genus (22).

L. reuteri has multiple beneficial effects on host health such as prevention or amelioration of diverse disorders (Figure 3) (23), improvement of nutrient absorption, modulation of host immune responses, promotion gut mucosal integrity and bacterial translocation reduction (24). Besides, it can inhibit the colonization and the growth of pathogenic microorganism Its antimicrobial activity is associated to the production of reuterin. It is known that *L. reuteri* can metabolize glycerol to generate 3-hydroxyproprionaldehyde (3-HPA) in a coenzyme B12-dependent glycerol dehydratase-mediated reaction (25). This metabolite is a potent anti-pathogenic compound, capable of inhibiting a wide spectrum of microorganisms including gram-positive bacteria, gram-negative bacteria, fungi, and parasites.

Both the EFSA (European Food Safety Authority) and the FDA (Food and Drug Administartion) have recognized its safety by authorizing its use also for products for children. There are several probiotic strains belonging to this species. Of human origin *L. reuteri* DSM 17938, *L. reuteri* NCIMB 30242, *L. reuteri* ATCC 6475.

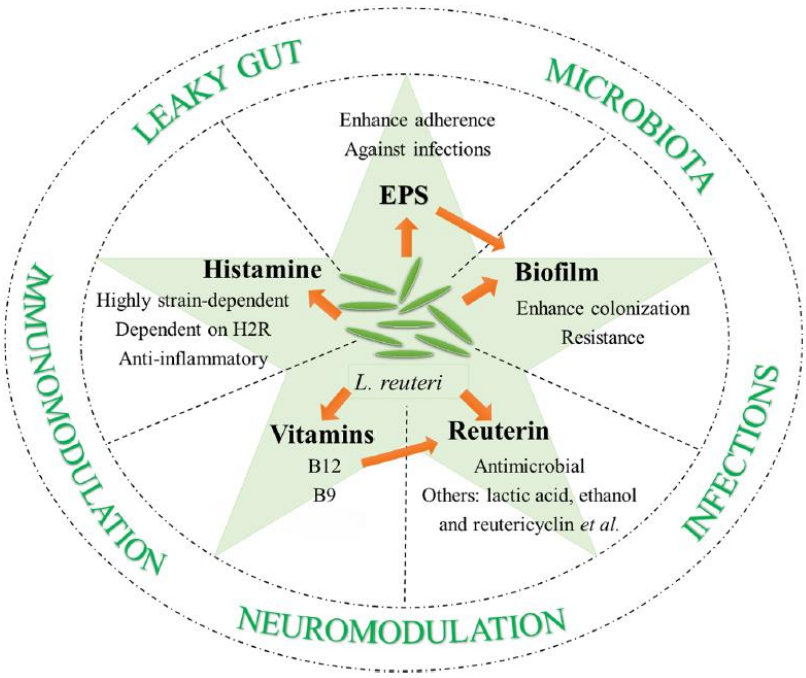

Figure 3: Probiotic properties of L. reuteri

1.3 Probiotic foods

Nowadays, consumer is looking for a diet for health and well-being, for this reason functional foods meet the needs of the consumer. So there is an increase of the consumption of novel functional food, either natural or processed foods (26).

Probiotic strains can be incorporated in food systems provided that certain safety and quality criteria are fulfilled: not be pathogenic; do not display toxicity; it must be resistance to gastric juice and bile salts with their ability to adhere to the intestinal mucosa (27).

The main target of probiotic food is the modulation of gut microbiota by inhibiting overgrowth of pathogenic bacteria through competition for adhesion sites and nutrients. The effectiveness of probiotic foods depends on survival during processing and storage of the food. So that probiotics

can manifest their beneficial effects they must also survive passage through the digestive tract and survive to bile salts. At the end, they must reach the intestine in a viable form so as to colonize and proliferate in it. Another fundamental aspect in the production of probiotic foods is the impact of the addition of culture on sensory characteristics (28). Furthermore, the minimum number of microorganisms that should be found at the end of the product shelf-life is, at least, 10^6 CFU/g of viable cells throughout (29).

In addition to the beneficial effects on the host, probiotics produce antibacterial species like bacteriocines, organic acids and hydrogen peroxide. In this way they can contribute to the prolongation of the shelf-life, due to the reduction of the growth of pathogenic bacteria Traditionally, probiotic foods belong to the dairy food category. However, the increase in lactose intolerance in the global population, the presence of allergens, the high cholesterol content, the recent scientific evidence of a negative correlation between the health of the individual and the consumption of milk and dairy products, and the spread of vegetarian or vegan diets have led to a reduction in the consumption of dairy products. Therefore, it becomes necessary to increase the accessibility and availability of probiotic products by identifying new food matrices that can act as carriers (Figure 4). Among these, the vegetable matrices, and in particular the fruit juices, seem to be able to satisfy the requests and needs of different groups of consumers.

Developing fruit juice that contains the desire probiotics will be a good alternative. Fruit juices have a pleasant taste that is acceptable by all age

groups. Furthermore, fruit juices contain beneficial nutrients, such as fibre, vitamins, antioxidants, minerals (30). Another advantage for fruit juices choice is their digestion in stomach faster than dairy products, so the probiotics spends much less time in the acidic environment of the stomach (16) (31).

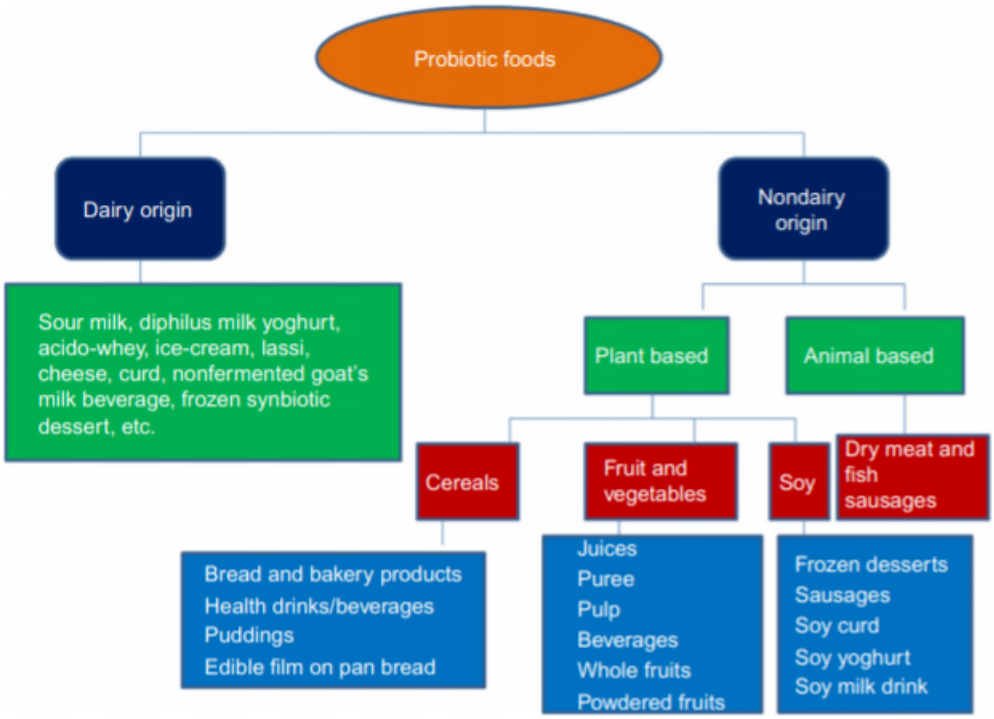

Figure 4: Classification of probiotic foods.

1.4 Tomato juice

Tomatoes originated in South America, in the general area of Peru and Ecuador, the Spanish took domesticated forms to Europe in 1523 and they had reached Italy by 1544.

The botanical name of tomato is *Solanum lycopersicum.* Tomato belongs to the family Solanaceae, which includes more than 3000 species. Tomato is one of the most widely grown and eaten food crops in the world, with an

annual global production of about 50 million metric tons (32). It is both consumed in fresh harvested form and in processed extracts form, such as tomato paste and tomato juice. Tomatoes are generally used as a vegetable, but botanically speaking they are fruit (33).

In raw red tomatoes are a low calorie food, with only 118 calories/100 g, with fat (1.49 g/100g), protein (8.81 g/100g), CHO (17.13 g/100g).Approximately 95% of the total lycopene is present in the all-trans form, it is located in the cell wall of the tomato, and red tomatoes (ripening) have more carotenoids (lycopene) comparing with green one (34).Tomatoes cooking in a bit of oil will increase the release of lycopene, so it has even greater bioavailability after cooking and processing (35) (36).

Tomato juice contains water (93.1%), carbohydrate (4.89%), carotenoids (10-20%)(37);Lycopene has the greatest contribution (around 83%) to the total pigments present in the tomato (38). Vitamins (high concentration of C, A and K) and minerals including potassium and manganese(39), it is low in protein and fat. Tomato juice is classified as healthy beverage.

Tomatoes help improve human health. There is scientific evidence about the correlation between tomato consumption and reduction of the risk of multiple conditions such as cancer, osteoporosis and cardiovascular disease. The red pigment of tomato (lycopene) has received most attention due to its antioxidant capacity with free radical scavenger activity. Epidemiological evidence suggests that diets rich in tomatoes and tomato

products decrease in cancer risk (40) (41). Sulphur in tomatoes protects the liver from cirrhosis. It can be used for healing sunburn because of its unique high content of vitamin C (20 mg/100 g).

The pH of tomatoes varies according to its ripening between pH 4.0 to 4.7, so it considers in medium acidic food category(33), spore-forming bacteria can be a risk factor, especially, mesophilic Bacillus *and Clostridium botulinum* (42) (43).

Tomato juice can be prepared by fully ripened red tomatoes (figure 5), a good quality juice should contain about 0.1% acid (in terms of citric acid) to avoid the danger of potential germination and growth of *C. botulinum* , 0.65% salt, 1% sugar, and 0.1% sodium benzoate as preservative(44). Tomato soluble solids content (Brix value) preferably to be around 7.5%. an average of 10 kg of tomatoes yields 7 litter of juice.

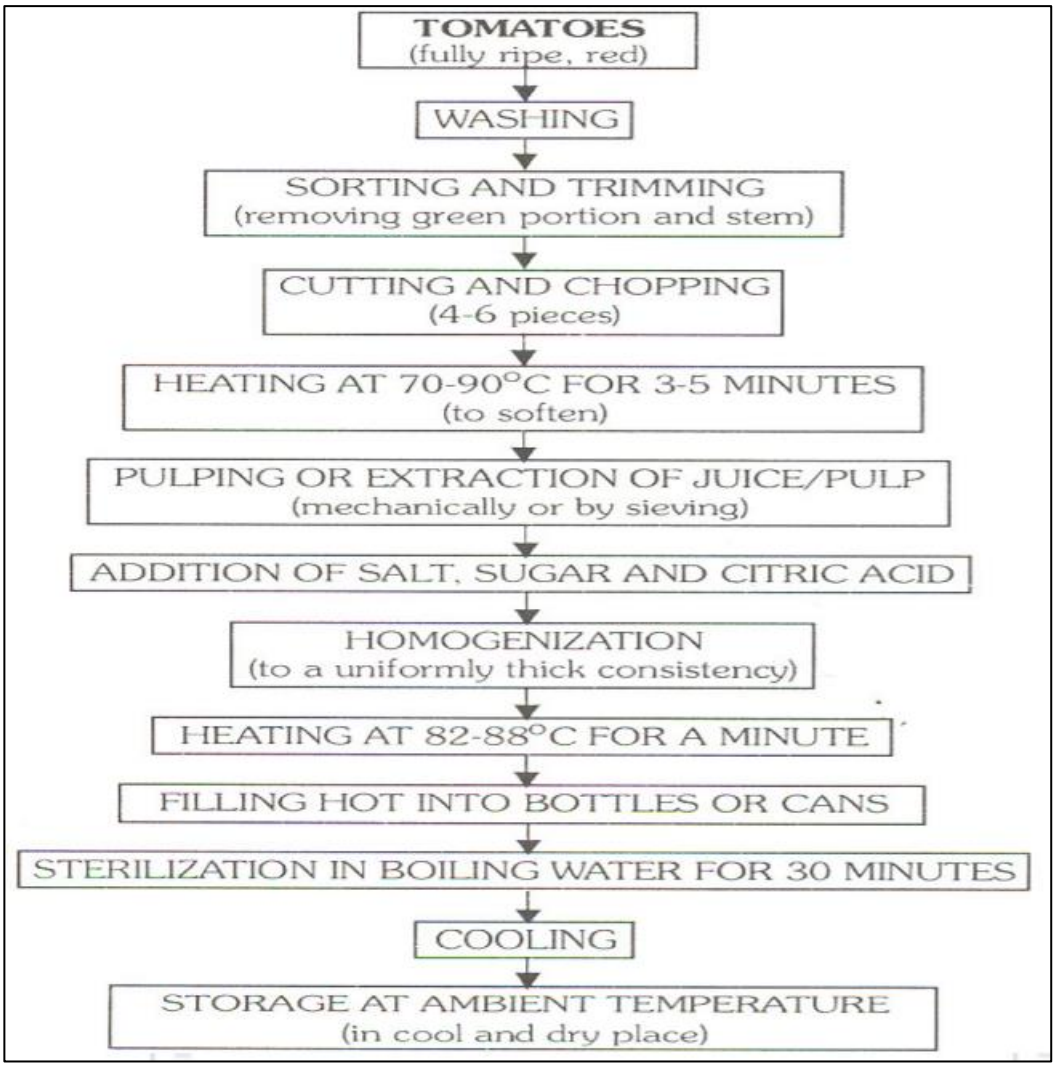

Figure 5: Processing flow sheet for tomato juice (44).

1.5 Attenuation.

Addition of probiotic to fruit juice leads to some modification on physico-chemical and sensory characteristics. These changes are the results of the fermentation process which means production of metabolites, especially organic acids. On one side this can help to increase the juice quality and its shelf-life. Many studies also showed the antioxidant effect of—the probiotication (27). On the other hand, the ability of the microorganisms to ferment sugars present in the fruit juice will consequently alter the sensory

profile. To minimize sensory changes, it is necessary to work with cultures with low metabolic activity.

Exposure of microbial cells to stress or sub-lethal adverse conditions induce a temporary or permanent alteration of their metabolism, with reference to the fermentative ability. Properly, this phenomenon is called attenuation.

Attenuation can be regarded as a tool to modulate the metabolism of probiotic bacteria and, consequently, a strategy to reduce the acidification of drinks. So attenuated lactic acid bacteria (LAB) cannot synthesize lactic acid during storage of probiotic foods, but in the same time, these attenuated cells have enhanced pool of intracellular enzymes released into the matrix that positively influencing the quality and flavour of final product, for this reason, researchers included this type of cell as starter cultures for cheese making to accelerate ripening and enhance light cheese flavour (45).

Attenuating treatment that reported in (1999) can be done through chemical and physical approaches.

- Chemical treatments: lysozyme, ethylenediaminetetraacetic acid (EDTA), isopropyl alcohol (IPA), dodecyl sulphate (SDS), hexadecyltrimethylammonium bromide (CTAB), alkanols like n-butanol.

Physical treatments: heating, freezing–thawing, spray and freeze drying, high pressure and high-pressure homogenization (300-700 MPa), microfluidization, sonication, microencapsulation.

1.5.1 Ultrasound

Ultrasound (US) is defined as a pressure wave with a frequency of 20 kHz or more generally, it uses frequencies from 20 kHz to 10 MHz that exceeds the hearing limit of the human ear. Ultrasounds are divided into low and high power ultrasounds, depending on the frequency and intensity of the wave.

Low power ultrasound:

- high frequency (> 100 kHz)
- low intensity (< 1 W / cm^2)

High power ultrasound:

- low frequency (20 - 100 kHz);
- high intensity (10 - 1.000 W / cm^2) (46).

Sonication needs two main requirements: liquid medium and source of high-energy vibrations. Generally, US is generated by electric energy supplied to a piezoelectric material (transducer), which converts the input energy into mechanical vibrations, and a fraction of energy is lost as heat which leads to reduce the efficiency of the treatment.

Sonication is generally applied to impart positive effects in food processing such as improvement in mass transfer; US provides a greater penetration of solvent into cellular materials, food preservation, assistance of thermal treatments and manipulation of texture and food analysis, this treatment is versatile and profitable to the food industry (47). In fact, this technique is used as preservation method. Due to its antimicrobial activity is used for ensure the quality and the safety of food. It was used in milk, dairy, meat, vegetables and fish due to its ability to inactivate the microorganism growth and enzymes (Table 2).

Ultrasonic waves have, also, the potential to exert a significant effect on microorganisms and living cells (45). In order to induce changes in microbial cells, high-power ultrasound is applied. The inhibition or inactivation of the probiotic is essentially the consequence of two physical phenomena: acoustic cavitation and acoustic flow. The sound waves, propagating in a liquid medium, determines pressure variations in the liquid itself. This leads to the creation of compression and expansion zones, with the formation of micro bubbles. The latter expand until they reach a maximum size. Upon reaching this state, the microbubbles implode, generating a local increase in temperature and pressure. This phenomenon, taken as a whole, is called acoustic cavitation. As a result of the previous events, the acoustic flow occurs. It consists in the dissipation of the acoustic cavitation energy through shear and turbulence energy. Secondly, the local increase in temperature and pressure leads to the formation of reactive species and free radicals.

The treatment applied can have direct consequences on microbial cells:

- attenuation of metabolism;
- increased permeability of the membrane, damage or destruction;
- morphological changes;
- DNA damage;
- damage to enzymes.

The efficiency depends on the power applied and the duration of the treatment, but above all on the interaction of these two variables. Therefore, it depends on the total energy dissipated in the system. Furthermore, different cells show a different sensitivity towards the treatment; spores > fungi (molds) > yeasts > gram-positive bacteria > gram-negative bacteria. The efficiency is, therefore, also dependent on the shape and size of the cell, The size is probably the leading factor for the different trends between fungi and bacteria because larger cells are generally more sensible because the surface exposed to US is greater(46) , as well as on the strain treated (48).According to the morphology cocci are more resistant than rods because of the relationship between cell surface and volume; according to their thicker cell wall Gram-positive bacteria are more resistant than Gram-negative one.For example, *Escherichia coli* and *Saccharmyces cerevisiae* were reduced by more than 99% after ultrasonication, where- as *Lactobacillus acidophilus* was reduced by 72%

and 84% depending on the media used, it also depends on the volume and composition of this sonicated food (49).

Low-intensity US stimulates bacterial metabolism and increases the transport of oxygen and nutrients to the deeper layer of biofilm. The stability of the biofilm and counteracted the detachment of cells from surfaces, Bacterial motility and adhesion are linked to various specific structures placed on the cell surface, such as flagella (linked to motility) and fimbriae (linked to adhesion); cellular stress, induced by an ultrasonic field, reduced flagella motility after 9 h of sonication at 67 kHz(50). An increase in biofilm formation and stability was also found for *Lactobacillus reuteri*. The positive effect on biofilm formation and adhesion could be linked to the increase of hydrophobicity and membrane permeability and these factors will enhance the adhesion of probiotic cells in gut as first stage, and adhesion to a specific cell-wall component of mucosa as the second mechanism

High-intensity ultrasound has been used for many years to generate emulsions, disrupt cells and disperse aggregated materials, it enhanced the microorganism aggregation ratio in high concentration, this leads to increase the initial cell number of bacteria after sonication; Physical, mechanical, or chemical effects of high intensity ultrasonic waves are capable of altering material properties (Figure 6) (e.g., disrupting the physical integrity, acceleration of certain chemical reactions) (51)

Table 2: Applications of US for some foods (46).

Food	Application
Milk and dairy beverages, rehydrated powder milk	*Inactivation of pathogens (milk, rehydrated powdered milk, infant formula, skim milk, and red-grape). *Homogenization of dairy beverages (probiotic milk, chocolate milk, whey-grape juice drink).
Juices and other beverages	*Inactivation of spoiling microorganisms and pathogens (orange, apple, pineapple, mango, red-fruits, and mulberry). *Inactivation of enzymes.
Vegetable and meat	*Inactivation of pathogens in fresh lettuce, cherry tomatoes, iceberg lettuce, celery, and fresh pepper *Improvement of meat tenderness (decrease of Warner-Bratzler force, increase of proteolysis, and improved disruption of myofibrillar structures).
Fish and shellfish	*Assisted extraction and fast determination of arsenic, selenium, vanadium, and nickel in fish and shellfish.
Table olives	*Enhancement of debittering in NaOH-free table olives

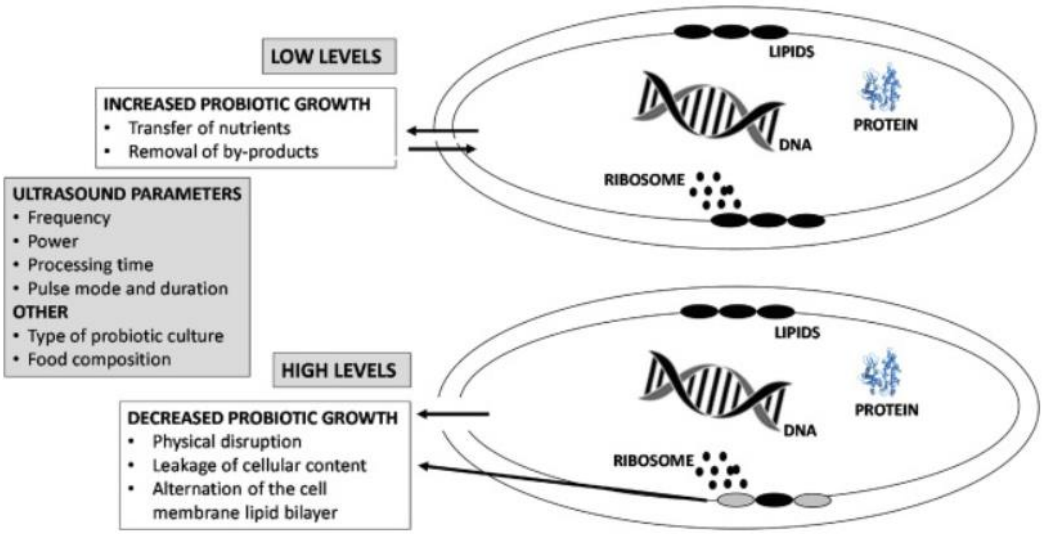

Figure 6: Effect of high-intensity ultrasound on the probiotic growth based on the sonoporation level (52).

1.5.2 Microencapsulation

In recent decades, there has been a rapid development of cell encapsulation systems. Encapsulation was one of the most widely used techniques in the food and pharmaceutical sectors; it can be obtained by immobilization in the matrix or through a simple coating. In the first case, we are talking about microencapsulation. It is defined as a process in which tiny particles (solid and liquid) or droplets are surrounded by a coating, or inserted within a homogeneous or heterogeneous matrix, in order to obtain small capsules with dimensions between millimeters and micrometers. Usually, polymers are used as a matrix. In order for the microcapsules to be added to food matrixes or for them to be used for drugs, it is necessary that the encapsulation materials are recognized as GRAS. Microcapsules, depending on the microencapsulation technique, have a spherical or irregular shape with a diameter of 1-1000 μm.

This technique can be applied for several purposes: protection of sensitive substances from the environment (UV rays, heat, O_2), targeted and controlled release, masking of unpleasant taste or odour, promoting easier handling, improving the process (Error! Reference source not found.**)** (53) (54).

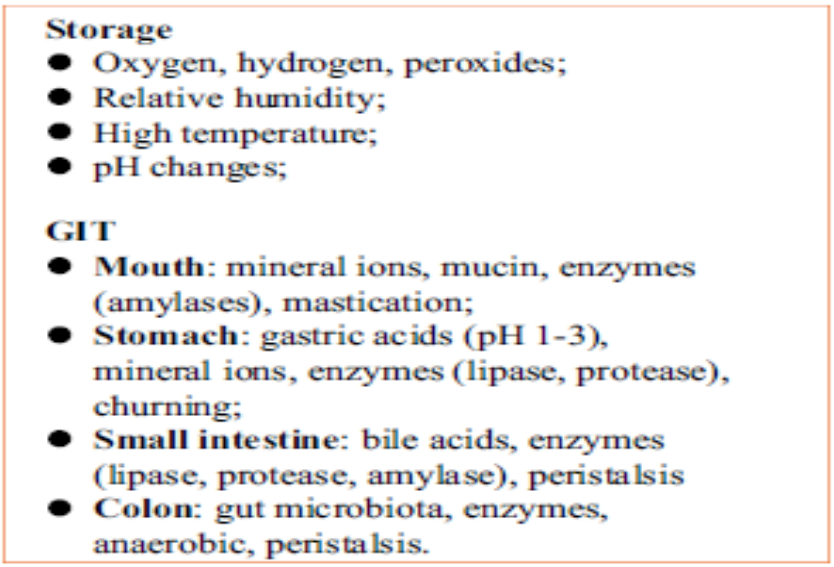

Figure 7: Harsh conditions probiotics encounter during storage and GI transit (53).

This technique is used to incorporate probiotic strains into functional foods. Although microcapsules are generally used as a transport system for probiotics, they can be used as an attenuation system. As a transport system, the microcapsule provides protection against external factors that can damage the probiotic. To provide human host beneficial action the strains should survive the passage through stomach and reach the intestine in sufficient quantities (between 10^6 and 10^7 CFU/ml). It can maintain the cells' growth and activate their metabolism in the intestinal tract and promote their ability to colonize the mucosal surfaces. As a method of attenuation, it must be considered that the microcapsules are surrounded by

a selectively permeable, thin but robust membrane, whose properties depend on the particular polymer used. Therefore, the bioactive component is physically separated from the external environment (food matrix) to which it is added in the form of a microcapsule.

This ensures that there are minimal interactions between the food matrix and the added probiotic microorganism. So, it’s possible to control metabolic activity and acidification of the food.

There are many techniques for microcapsules formations such as: emulsion, extrusion, coacervation, spray drying, ultrasonic vacuum spray dryer, freeze drying, spray-freeze drying, spray chilling (also known as spray cooling or spray congealing), fluid bed coating, electro-spraying – electrospinning, impinging aerosol technology (55).

Spray drying is the most common technology that used in the food industry due to its low cost, the availability of equipment, high reproducibility and high rate of production, but it has high cell morality rate due simultaneous dehydration and thermal inactivation of microorganisms (56).

Vibration technology a new technique for the production of different types of microcapsules for application in biotechnological processes. It based on breaking up a laminar liquid stream into droplets by a superimposed vibration. These are collected in a gelling bath with consequent solidification of the coating material. The microcapsules formed are then collected by sedimentation or centrifugation. The fundamental parameters for applying the technique are:

- nozzle size;
- distance between nozzle and gelling bath;
- concentration, T, viscosity, flow rate of the polymer solution;
- capsule size (depending on the parameters listed above; generally, between 2-5 mm).

Vibration technology has gained a significant interest mainly due to the capability to produce uniform and monodisperse microspheres.

Any encapsulator that works on vibrating technology includes four main items; i) feeding system to inject polymer-product by using syringe and this feeding regulated by the pump function, ii) Pulsating chamber and nozzle that pumped the mixture, droplet forms from mixture linear flow that broken from vibration of membrane, the number of vibration regulated by controlled the frequency parameter, iii) Magnetic field that generated between the nozzle and electrode that leads to the charging the surface of droplets, electrostatic repulsion forces results between droplets that cause the dispersion of the beads, this field regulated by the electrode parameter, iv) Gelling polymer that uses for beads hardening, finally microcapsules are subsequently recovered (figure 8).

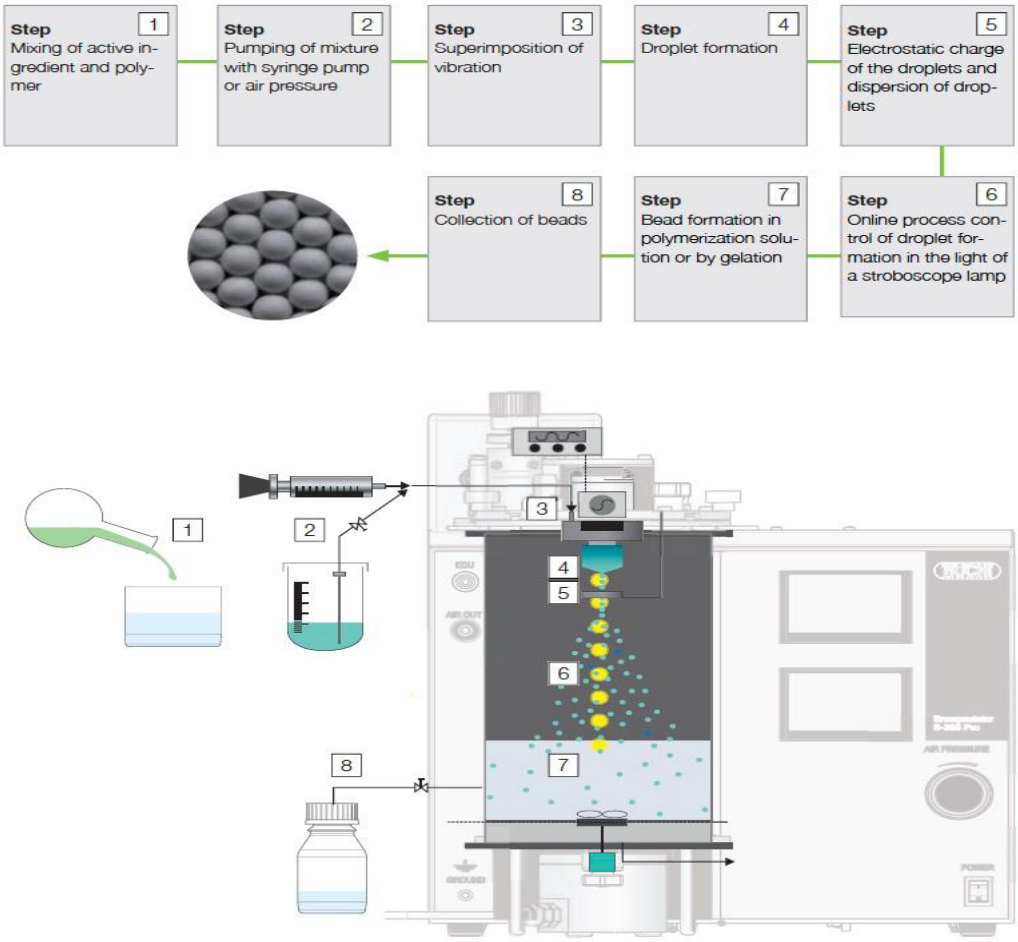

Figure 8: Vibrating technology for microencapsulation (57).

The microcapsules should be stable and preserve its integrity during the storage and digestive tract passage until it reaches at its target destination, where the capsules should release its bioactive contents (58).

Many coating polymers are used for protection and strength barrier target as an encapsulation matrix for probiotic formulation, such as alginate, starch, gelatin, xanthan gum, etc.

1.5.2.1 Alginate

Alginate is the most popular encapsulation material in probiotic foods (Figure 9). Alginate is produced by the bacterial genera *Pseudomonas* and *Azotobacter*, and by marine brown algae.

It is a linear polysaccharide consisting of β-(1→4)- linked D-mannuronic acid (M) and α-(1→4)-linked L-guluronic acid (G) residues (59), natural copolymer with a non-regular block- wise arrangement between the G and M units.

In the presence of divalent metal ions; such as Ca^{+2} in form of calcium chloride ($CaCl_2$), some alginate solutions can form a gel by forming junction zone what has been described as an “egg box structure” between four G residues (Figure 10), that is described as calcium ion being held in ionic bridges formed between Ca^{+2}ions and the ionized carboxyl groups of adjacent alginate chain, in another words, calcium ions induce chain-chain association (60).

It is an anionic polysaccharide, due to the presence of carboxylic groups, biocompatible, biodegradable, soluble in water at temperatures of 60-80 ° C, and is characterized by low toxicity, low cost, thermo-stability. It is recognized the status of GRAS by the FDA. For these reasons Alginate has a great potential for application in the pharmaceutical and food fields, it suitable for use both as a conventional excipient and in drug encapsulation and transport systems (61).

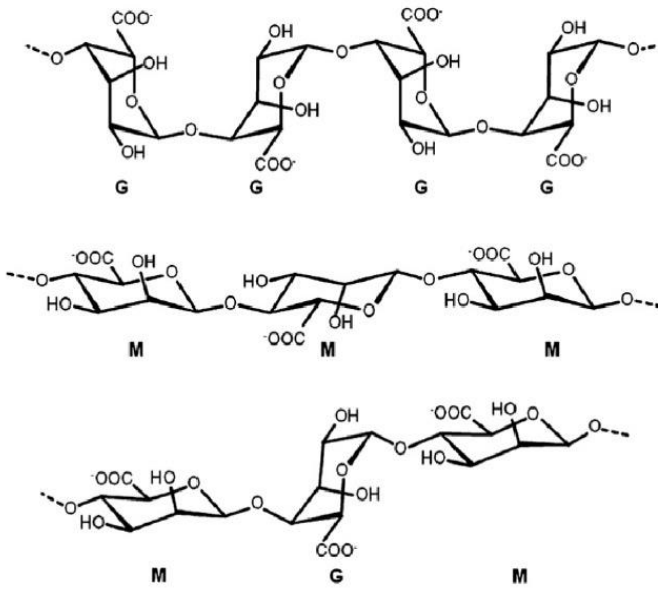

Figure 9: Chemical structures of G-block, M-block and polymerization of alginate(62).

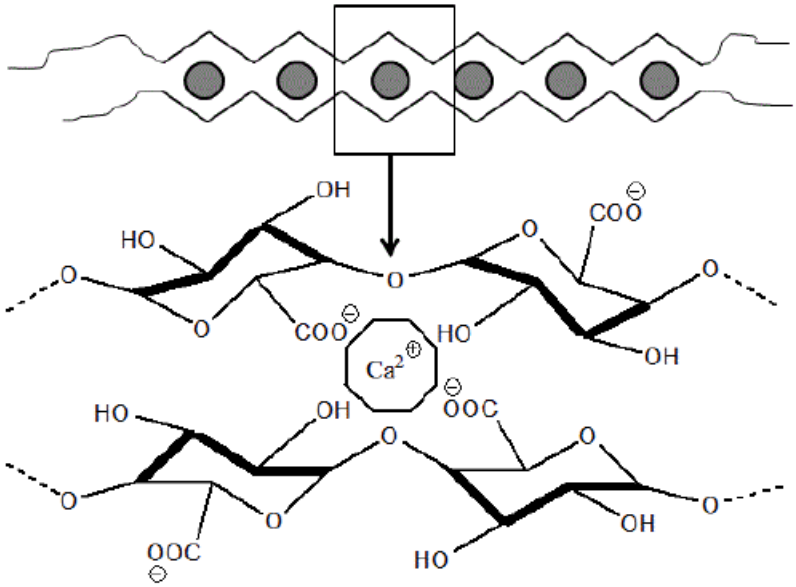

Figure 10: Schematic picture illustrating the egg-box model for Ca^{+2} alginate(60).

1.6 Hypothesis of this work

This work based on a hypothesis that attenuated probiotic culture *Limosilactobacillus reuteri DSM 17938(L. reuteri)* that inoculated in tomato juice with controlling the pH and viability of *L. reuteri* reduction. In addition, sensory attributes changes should be controlled after probiotics metabolism and carbohydrates fermentation during 28 days at (4^{o}- 20^{o}) C. *L. reuteri* will be attenuated using ultrasound technique(US), US is to reduce metabolic activity of these probiotics. So we have to identify the best combination between power (W) and time (Min) of sonication to get the minimum reduction value for both of pH and viability of *Limosilactobacillus reuteri DSM 17938*, microencapsulation of *L. reuteri* was used as a part of attenuation to get our targets in this study.

1.7 Objectives

From the studies in the literature, there is need to expand the types of probiotic foods is evident, guaranteeing the possibility of using them also to groups of consumers with specifics needs.

The objective of this thesis project is, therefore, to identify a new food matrix suitable for probiotication, that is, such as to guarantee the survival of the microorganism during storage. Equally important is obtain a product with chemical-physical and sensory characteristics similar to the traditional one, or however acceptable to the consumer. For this reason, one of the cornerstones of the thesis project is application of physical treatments,

known as attenuation treatments, aimed at modulating the influence of probiotic on the food matrix.

Chapter Two
Material and Methods

2.1 Strain and preparation of culture media

Limosilactobacillus reuteri DSM 17938 was isolated from Reuflor ® (BioGaia) and cultured in MRS broth (*Oxoid, Milan, Italy*) at 37 °C for 24 h.

To cultivate L. reuteri DSM 17938 in plates it has been used Tryptone Soya Agar (TSA) with the addition of 0.5 % of Yeast Extract. All these ingredients from (*Oxoid, Milan, Italy*). The media were sterilized by autoclaving (121 °C, 15 min).

2.2 US-treatment and acidification

After incubation for 24 h in MRS broth, the cells were centrifuged at 4500 rpm for 10 min, the supernatant were discarded and the pellet were re-suspended in equal volume of deionized water. A volume of 30 ml was used for sonication. Bacteria cultures were sonicated through LABSONIC U (B Braun). Overall, the treatments carried out were 6. The three main parameters were: the power level; the duty cycle; the time of treatment.

The duty cycle remained unchanged in the different tests and set at 0.5, while the power level and duration were changed. The power level was set at 57 (treatment A), 64 (treatment B) and 78 W (treatment C). For each power level the samples were treated for 4 and 6 min (Table.3). Before each treatment, the ultrasonic probe was washed with 70% ethanol. After

sonication, the samples were placed in ice box due to the increase of temperature.

After sonication treatment, the sonicated cell suspension and the untreated microorganism were inoculated in MRS broth (inoculum at 1%). The untreated bacterium was used as control. The samples were incubated at 37^{o}C for 48 h. The pH of the medium was measured through the pH-meter (BENCH METER-pH 80) (Figure 11) at time zero, and after 6 and 24 h of incubation (63).

Figure 11: pH meter.

2.3 Effect of ultrasound on viable count

Viable count was determined before and after sonication. The microorganism was grown according to the spread plate method. First, a series of serial decimal dilutions was set up by using Ringer's solution. Then, 100 μl were taken from each tube and transferred to Petri dishes

containing TSA. Plates were incubated 37 °C for 48h. The results were reported as CFU/ml.

2.4 Effect of ultrasound on some functional properties

After sonication (57 W, 6 min), other experiments were done directly. The effect of ultrasound was tested on some functional properties like survival in the presence of bile salts and survival at low PH.

A solution of 0.15% bile salts was prepared. The sonicated cell suspensions were centrifuged (4,000 rpm, 10 min) (Figure 12). The pellet was re-suspended in equal volume of bile salts solution, after discard the supernatant. Then the samples were incubated at 37 °C for 3 h. A serial of decimal dilutions was made and the viable count was defined. The untreated culture was use as control. The samples were incubated at 37 °C for 24 h.

For the survival at low pH, it was used an acidified deionized sterile water (pH 2.5). We then proceeded as above.

Figure 12: **Centrifugator for bacterial culture**.

2.5 Chemicals preparation for microencapsulation

Microencapsulation of *L. reuteri* DSM 17938 were done with a solution of sodium alginate. The solution was made by dissolving the powder of sodium alginate by heating (*Sigma, Milan, Italy*) in deionized water (1,2 %). Then, the alginate solution was filtered with gauze to remove any undissolved particles to avoid closing nozzle of microencapulator.

For the solidification on microcapsules was used a solution of $CaCl_2$ (0.5 M).

The solutions were sterilized by autoclaving (121 °C, 15 min).

2.6 Encapsulation of L. reuteri DSM 17939

Microencapsulation of sonicated bacterial cells was carried out by using the Encapsulator B-395 Pro equipped with 120 mm nozzle and a syringe pump (BÜCHI Labortechnik, Flawil, Switzerland) (Figure 13).

After sonication, the cell suspension of *L. reuteri* DSM 17938 were centrifuged at 4,500 rpm for 10 min. The cell pellet was washed twice with Ringer solution and finally suspended in equal volume of sodium alginate. The alginate cell suspension was loaded in 50 ml syringe and then placed on the Encapsulator according to the instruction of supplier.

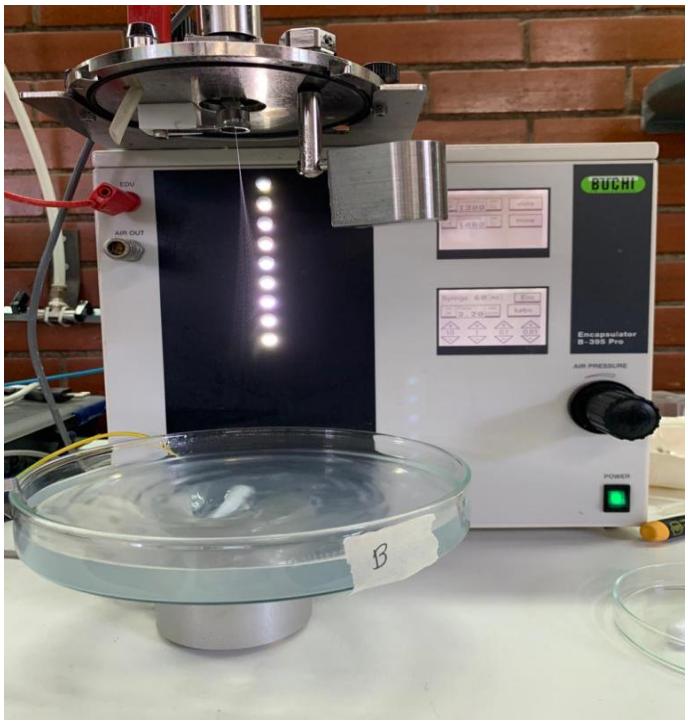

Figure 13: Encapsulator B-395 Pro equipped with 120 µm nozzle and a syringe pump.

Encapsulation parameters used: vibration frequency 1300 Hz, electrode voltage1800 mV. Alginate cell suspension were hardened in 0.5 M $CaCl_2$ solution for about 20 min in stirring to obtain monodisperse cross-linked

microcapsules. The microcapsules were collected after sedimentation (Figure14). Two separated phases were resulted, the upper phase that contained $CaCl_2$ solution was removed and discarded using sterile pipette.

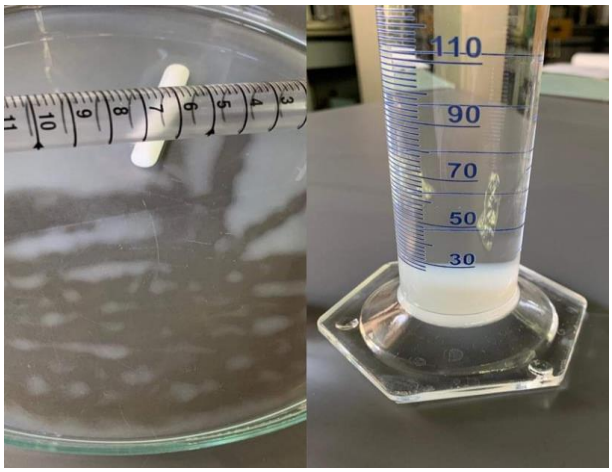

Figure 14: L. reuteri DSM 17938 capsules collection.

2.7 Probiotication of tomato juice

A commercial tomato juice was purchased from Carrefur market (Figure.

The probiotication of tomato juice was done in three different ways:

- with a cell suspension;
- with a sonicated cell suspension;
- with microcapsules of the sonicated cell suspension.

Figure 15: Nutritional table for tomato juice that inoculated.

2.7.1 Probiotication of tomato juice with free L. reuteri DSM 17938

A fresh culture of *L. reuteri* DSM 17938 was centrifugated at 4000 rpm for 10 min. The pellet was washed twice with Ringer solution and suspended in an equal volume of tomato juice.

The cell suspension in tomato juice (LR) was then added in falcons with tomato juice to reach a concentration of 10^7 CFU/ml (Figure 16).

The samples were stored at 4 and 20 °C for 28 days.

2.7.2 Probiotication of tomato juice with sonicated L. reuteri DSM 17938

The ultrasound treatment was done as describe above (57 W, 6 min). The sonicated cell suspension was centrifugated (4500 rpm, 10 min) and washed twice. The supernatant was discarded and the pellet were suspended in equal volume of tomato juice.

The cell suspension in tomato juice (LR-US) was then added in falcons with tomato juice to reach a concentration of 10^7 CFU/ml, the samples were stored at 4 and 20 °C for 28 days.

2.7.3 Probiotication of tomato juice with microcapsules of sonicated L. reuteri DSM 17938

After sonication, the cell suspension was centrifuged (4500 rpm, 10 min) and then washed twice. The pellet was suspended in equal volume of sodium alginate solution and microencapsulated as describe above (section 2.7). After sedimentation, the microcapsules (LR-US-MC) were collected and placed into sterile falcons. Then the tomato juice was added to reach the original volume of 30 ml. Finally, the microcapsules in tomato juice were added in falcons with tomato juice to reach a concentration of 10^7 CFU/ml**,** the samples were stored at 4 and 20 °C for 28 days.

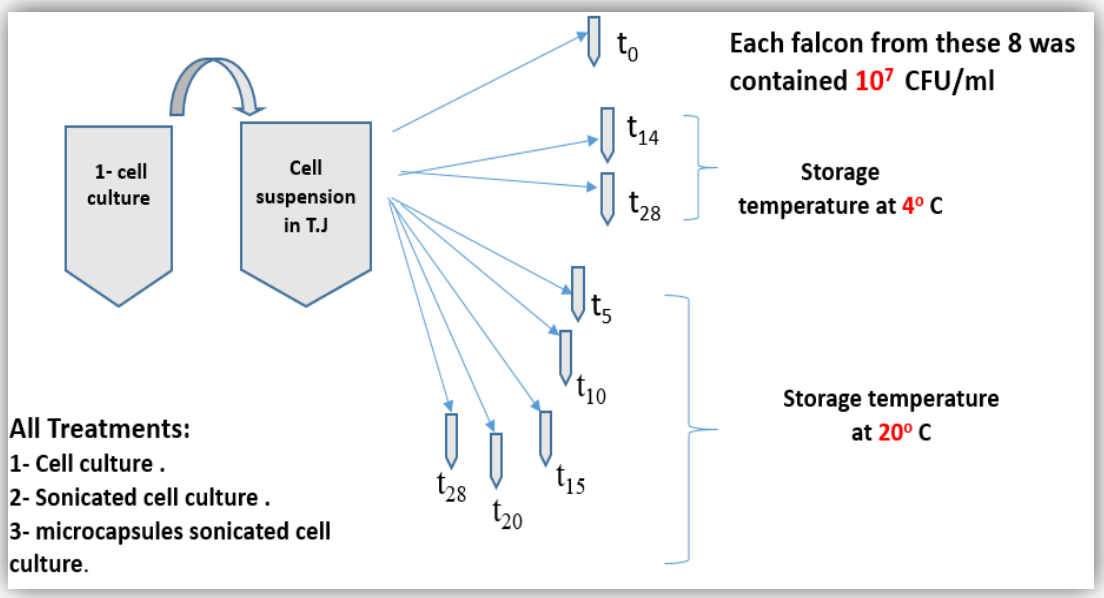

Figure 16: scheme of probiotication tomato juice

2.8 pH and viable count during storage

During the storage the pH and viable count were evaluated. The samples stored at 4 °C have been assessed for the pH and the viable count every 14 days. Instead, the samples stored at 20 °C have been assessed for the pH and the viable count every 5 days.

The viable count was done as describe above (section 2.4). But for the microcapsules, the serial decimal dilutions were made with the first tube of a solution of sodium citrate (0,2 M) to allow the disruption of the microcapsules that leads to bacteria release.

2.9 Sensory Analysis

The samples added with the microorganism in free form and with the microcapsules stored at 4 ° C were subjected to sensory analysis. A panel of trained tasters was used for the analysis (n = 30). The panel is made up of male and female individuals with an average age of 31 years. The evaluation was carried out using the “difference from control” test. During the test 4 samples (15 ml per sample) were presented to each panelist: the reference, that is the commercial tomato juice, a juice sample with the probiotic in free form, a juice sample with the probiotic in the form of microcapsules and a sample of commercial juice (as a hidden reference) (Figure 17). Each sample was marked with a random three-digit numerical code. Except for the reference marked “R”. The taster is therefore requested to indicate whether or not there are differences between the samples and the reference through a scale ranging from 0 (equal to the reference) to 10 (completely different from the reference). In addition, they were asked to list which are the attributes for which the sample is perceived different. The test and data collection were carried out using the Fizz software.

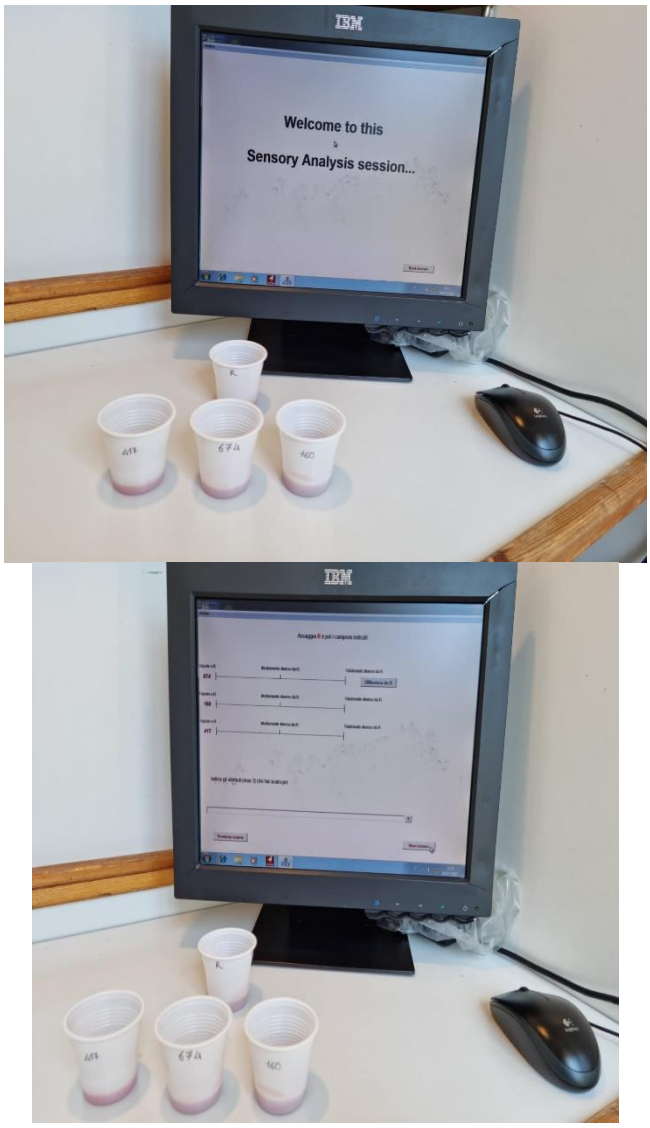

Figure 17: Sensory analysis using the Fizz software.

2.10. Statistical analysis.

All treatments were carried out three independent batches. In each case, an untreated sample acted as a control. Microbial populations were transformed into log values. Two-wayANOVA procedure for balanced design, and Bonferroni test for multiple comparisons of means, analysis were conducted using the Statistical Analysis System (IBM-SPSS) software.

Chapter Three
Results

3.1. Effect of sonication on L. reuteri DSM 17938

3.1.1 Effect on acidification and viable count

The effect of sonication was preliminarily evaluated on two aspects of the microorganism: fermentative metabolism and viability. Sonication must cover two basic targets: avoid and/or slow down post-acidification, that results by *L. reuteri* DSM 17938 metabolism, without affecting its viability. So, the first target was aimed to choose the optimal combination of power level and duration time of sonication that is able to achieve these two targets.

Table 3: Decrease of pH in MRS broth inoculated with L. reuteri DSM 17938; the measurements were done after 6h at 37 °C. The reported values derive from the average of the collected data (mean values ± SD, n=3)

		Power (W)	
	57	64	78
Sample			
NT-LR*	1.09 ± 0.03a,[1]	1.03 ± 0.06a	1.00 ± 0.18a
LR-US / 4 min**	0.63 ± 0.18a	0.73 ± 0.08a,b	0.25 ± 0.06c
LR-US / 6 min	0.08 ± 0.03a	0.03 ± 0.08a	0.00 ± 0.01a

**Not-treated L. reuteri DSM 17938.*

***Treated (sonicated) L. reuteri DSM 17938.*

[1]The same letters in the same raw indicate that the differences are not significant for each duration time and each power, (Two-way ANOVA and Bonferroni test for multiple comparisons, P >0.05).

Table 3 shows the effect of *L. reuteri* DSM 17938 addition, before and after sonication, on the pH reduction (ΔpH) of the inoculation medium (MRS broth) after 6h of incubation at 37 °C.

The results are expressed as pH reduction from t_0 to t_6 and t_{24}.

From the results obtained it is clear that an increase in power and in duration of treatment leads to a decreased of acidification in case of t_6.

Table 4: Decrease of pH in MRS broth inoculated with L. reuteri DSM 17938; the measurements were done after 24h at 37 °C. The reported values derive from the average of the collected data (mean values ± SD, n=3)

Power (W)						
		57		64		78
Sample						
NT-LR*		2.06 ± 0.07a,[1]		1.98 ± 0.08a		2.08 ± 0.08a
LR-US / 4 min**		2.04 ± 0.04a		1.95 ± 0.08a		2.10 ± 0.16a
LR-US / 6 min		2.04 ± 0.03a		1.96± 0.08a		2.06 ± 0.13a

**Not-treated L. reuteri DSM 17938.*

***Treated (sonicated) L. reuteri DSM 17938.*

[1] The same letters in the same raw indicate that the differences for each duration time and each power are not significant, (Two-way ANOVA and Bonferroni test for multiple comparisons, P >0.05).

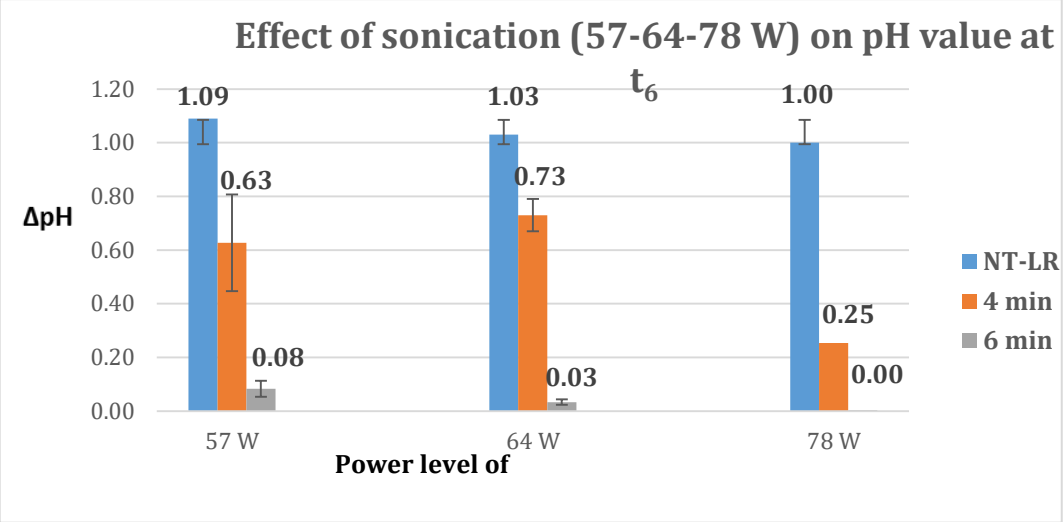

Figure 18: Effect of all sonication combinations on post-acidification by L. reuteri DSM 17938 at t_{24} compared with un-treated sample as control.

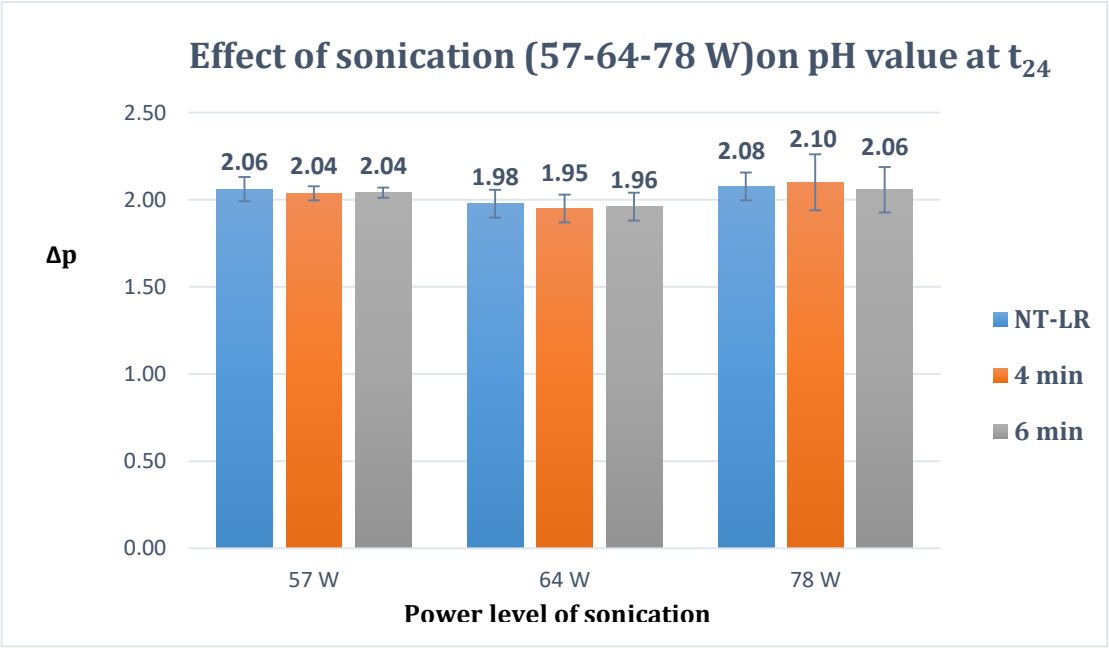

Figure 19: Effect of all sonication combinations on post-acidification by L. reuteri DSM 17938 t_{24} compared with un-treated sample as control.

Regardless of the parameters set, the effect of sonication on the fermentative metabolism is momentary. In fact, after 6 h of incubation at 37 ° C between the untreated sample and the sonicated cells there are significant differences($P<0.05$), as shown in Figure 18, which are not significant after 24 h of incubation under the same conditions ($P > 0.05$), as

shown in Figure 19. Probably, the transient effect of sonication is due to the denaturation induced by ultrasound of the enzymes responsible for the fermentation of sugars (64).

Figure 19 shows the overall results of ΔpH values at t_{24} after sonication using three different power levels for two duration time (4 and 6 min). It is approved that minimal reduction pH at power level 78W compared with two other power levels.

Table 5: Viability of L. reuteri DSM 17938 immediately after US-treatment with 6 combinations and of untreated culture (NT-LR). The reported values derive from the average of the collected data (mean values ± SD, n=3)

Power (W)			
	57	64	78
Sample	log CFU/ml	log CFU/ml	log CFU/ml
NT-LR	9.40 ± 0.02a	9.16 ± 0.11a	9.23 ± 0.09a
LR-US / 4 min	8.99 ± 0.05a	8.74 ± 0.08a	8.67 ± 0.16a
LR-US / 6 min	8.90 ± 0.04a	7.65± 1.00b	6.32 ± 0.10c

[1] The same letters in the same raw indicate that the differences for each duration time and each power are not significant, (Two-way ANOVA and Bonferroni test for multiple comparisons, P >0.05).

The second basic requirement is the viability. Thus the 6 combinations able to avoid acidification were tested and the viable count was determined before and after the sonication.

Treatment B and C caused a highly significant reduction of cell count 2.91 log CFU/ml and 1.51 log CFU/ml at duration time 6 min, respectively ($P<0.05$). In contrast, treatment A for 6 min reduction was only 0.5 log CFU/ml resulted on viability as shown in Table 5.

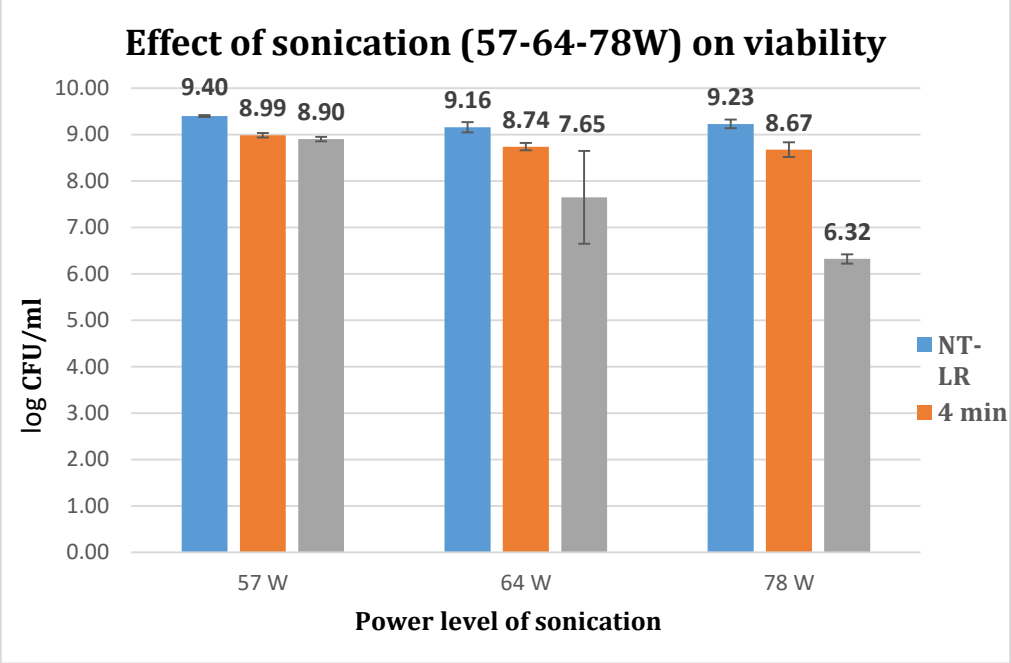

Figure 20: Effect of sonication and duration time combinations on viability of L. reuteri DSM 17938 compared with un-treated sample as control.

From the data collected, it appears that treatment A at power level 57W for 6 min allows to achieve the set goal (Figure 19 and Figure 20**).** Since the attenuation induced by sonication is only temporary, it is necessary to further intervene on the crop to control its fermentative metabolism. The choice of using the combination of several technologies could be beneficial.

According to the acceptable results for the viability of *L. reuteri* DSM 17938 and its post-acidification, it was chosen as the best combination to test the effect of sonication on cell morphology and on some functional properties.

3.1.2 Effect on morphology

Ultrasound, in addition to the attenuation of metabolism and the reduction of vitality, are responsible for various affecting the plasmatic membrane. These variations include increased membrane permeability, damage or destruction of the membrane and morphological changes.

To study how sonication affects the morphology of the *L. reuteri* DSM 17938 an observation under the optical microscope was made.

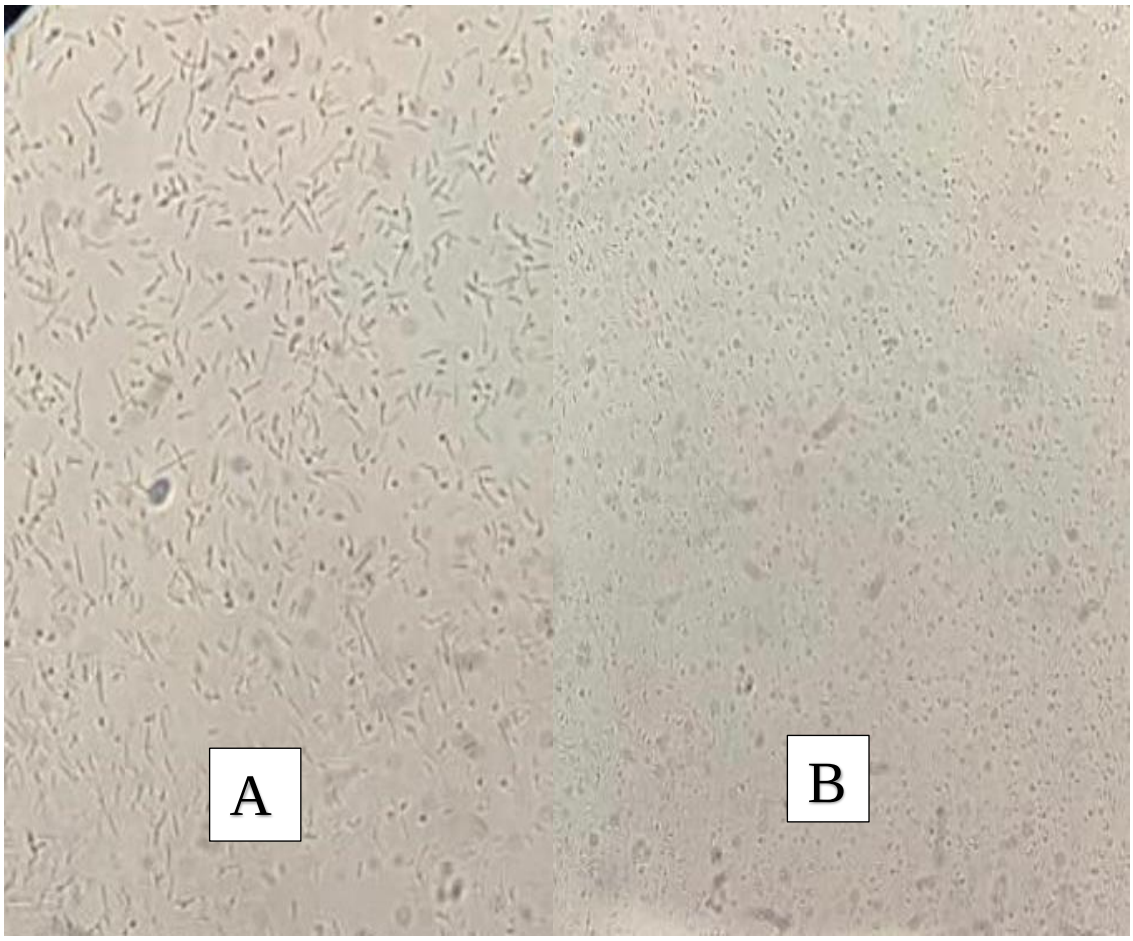

Figure 21: photos of microscopy in case of un-treated L. reuteri (A), sonicated L. reuteri (57 W) for 6 min (B).

As shown in Figure 21**,** the morphology of the probiotic was affected after sonication. In fact, before the treatment, the microorganism appears in the form of sticks. Following sonication, however, the cells appear visually smaller. The combination 57 W/6 min can be considered intense treatment, so it leads to physical disruption and alteration of cell membrane (52)

3.1.3 Effect on functional properties

After choosing the most suitable power/duration combination according to the intended objective, the influence of the ultrasounds was investigated on some probiotic traits of the strain in question.

Some preliminary experiments were done to check the effect of sonication on *L. reuteri* DSM 17938, survival at low pH and survival in presence of bile salts.

Table 6: Decrease of viability after 3 hours of incubation in deionized sterile water pH 2,5 or in deionized sterile water containing 0,15 % of bile salts. CNT: control non treated; TR: microorganism treated with ultrasound. The reported values derive from the average of the collected data (mean values ± SD, n=3).

Reduction of logarithmic cycles log CFU/ml	
CNT* - pH 2.5	0,91 ± 0,04
TR** - pH 2.5	2.30 ± 0.11
CNT - BS	6.12 ± 0.10
TR - BS	3.29 ± 0.03

*Control-Not Treated (Not Sonicated).

**Treated (sonicated) samples.

Table 6 shows the significant reduction ($P<0.05$) in viability for non-treated and treated probiotic strain after 3 h of incubation at 37 °C under the conditions described above.

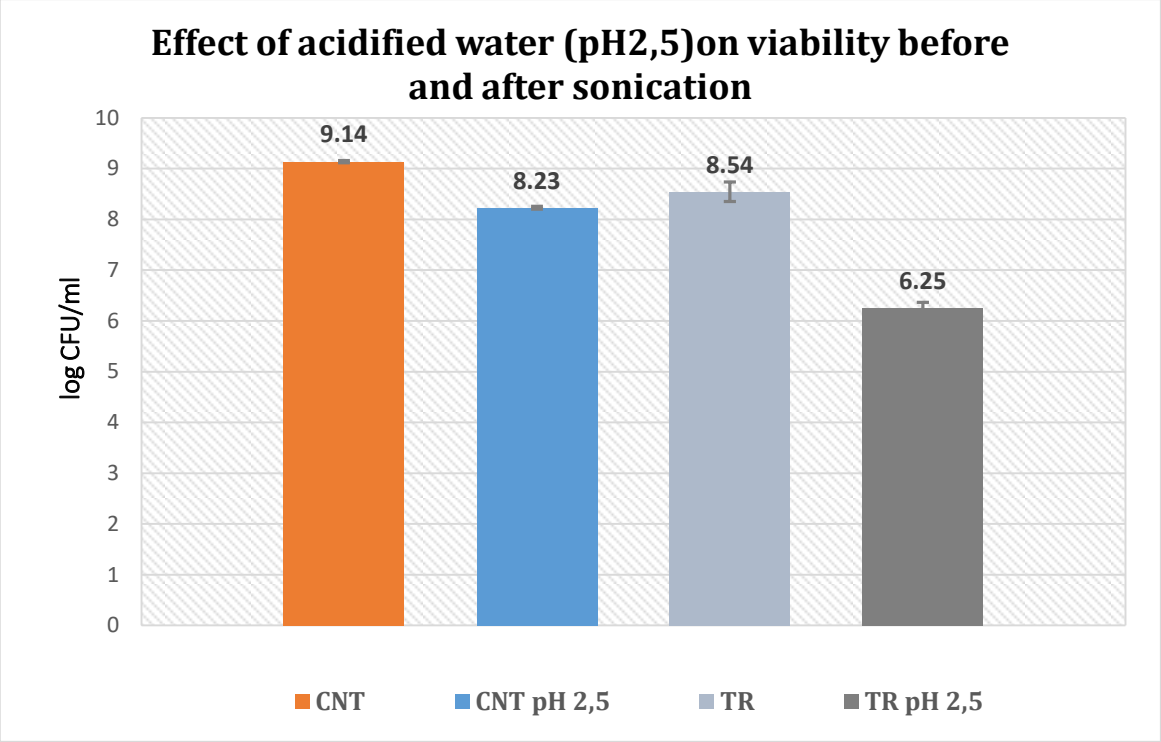

Figure 22: Effect of acidified sterile water pH 2,5 on the viability of L. reuteri that inoculated on this water before and after sonication at (57 W/ 6 Min) , un-treated samples were used as control.

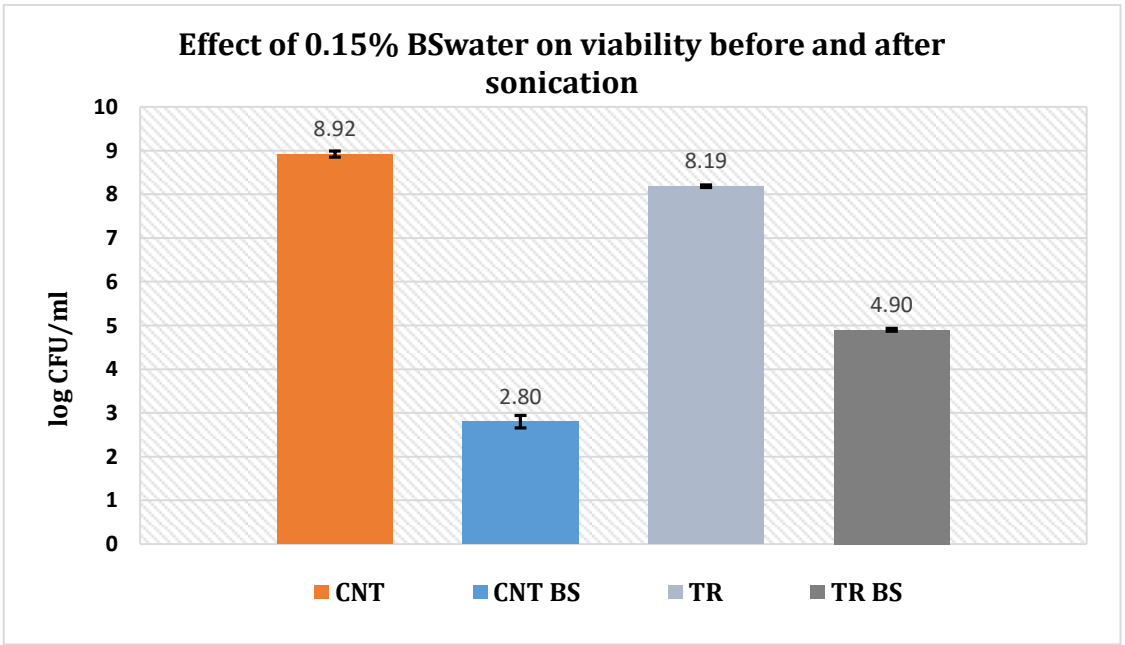

Figure 23. Effect of 0.15% BS sterile water on the viability of L. reuteri that inoculated in this water before and after sonication at (57 W/ 6 Min), un-treated samples were used as control.

At low pH 2.5 *L. reuteri* DSM 17938 has a higher survival than the treated one.

The significant reduction (P<0.05) that occurred after US treatment was greater. So, sonication increases the probiotic's sensitivity at pH 2.5 as shown in Figure 22**.** In contrast, the signinficant reduction of viability (P<0.05) in case incubation in deionized sterile water containing 0.15% of bile salts of non-treated *L. reuteri* DSM 17938 was higher than half sonicated microorganism as shown in Figure 23. The actual explanation of this results is not addressed very well in literature; more studies is required to get more data on this.

3.2 Microencapsulation of L. rueteri DSM 17938

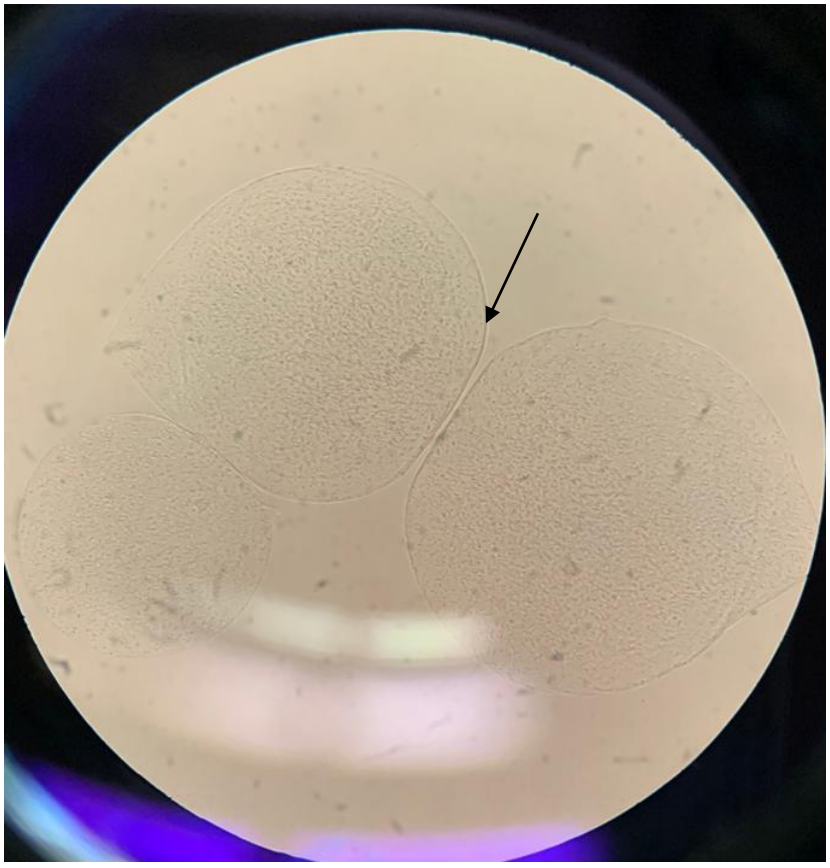

Figure 24: photo for L. reuteri capsules.

Capsules of *L.reuteri* with diameter around 240 µm with continuous surface of cell membrane were resulted from nozzle with diameter size (120 µm) that was used for microencapsulation using vibrating technology (Figure 24), the literature approved that the size of capsules will be larger than the diameter of nozzle that used **(57).**

3.3 Storage of probiotic tomato juice

Table 7: pH-values for three different probiotic tomato juice samples during the storage time at 4° C.

Time (days)	Samples		
	LR	LR-US	LR-US-MC
0	4.24 ± 0.03a,[1]	4.28 ± 0.04a	4.18 ± 0.07a
14	4.27 ± 0.06a	4.24 ± 0.10a	4.20 ± 0.06a
28	4.36 ± 0.01a	4.26 ± 0.06a	4.13 ± 0.04a

[1] The same letters in the same raw indicate that the differences for each storage time and each sample are not significant, (Two-way ANOVA and Bonferroni test for multiple comparisons, P >0.05).

Table 8: The viability of L. reuteri (log CFU/ml) in case of three different probiotic tomato juice samples during the storage time at 4° C.

Time (days)	Samples		
	LR	LR-US	LR-US-MC
0	7.14 ± 0.08[a]	7.15 ± 0.20[a]	7.63 ± 0.09[a]
14	6.97 ± 0.14[a]	6.27 ± 0.72[a]	7.43 ± 0.00[b]
28	5.70 ± 0.28[a]	5.94 ± 0.50[a]	7.24 ± 0.04[b]

[1]The same letters in the same raw indicate that the differences for each storage time and each sample are not significant, (Two-way ANOVA and Bonferroni test for multiple comparisons, P >0.05).

The three types of probiotic tomato juice LR, LR-US, LR-US-MC (section 2.7) were stored at 4 and 20 °C for 28 days. During storage, the juices were characterized for two parameters: the pH and viability of the added microorganism.

Table 7 and Table 8 show the results obtained from pH monitoring and the results obtained from the plate count respectively. These tests were done from time zero every 14 days.

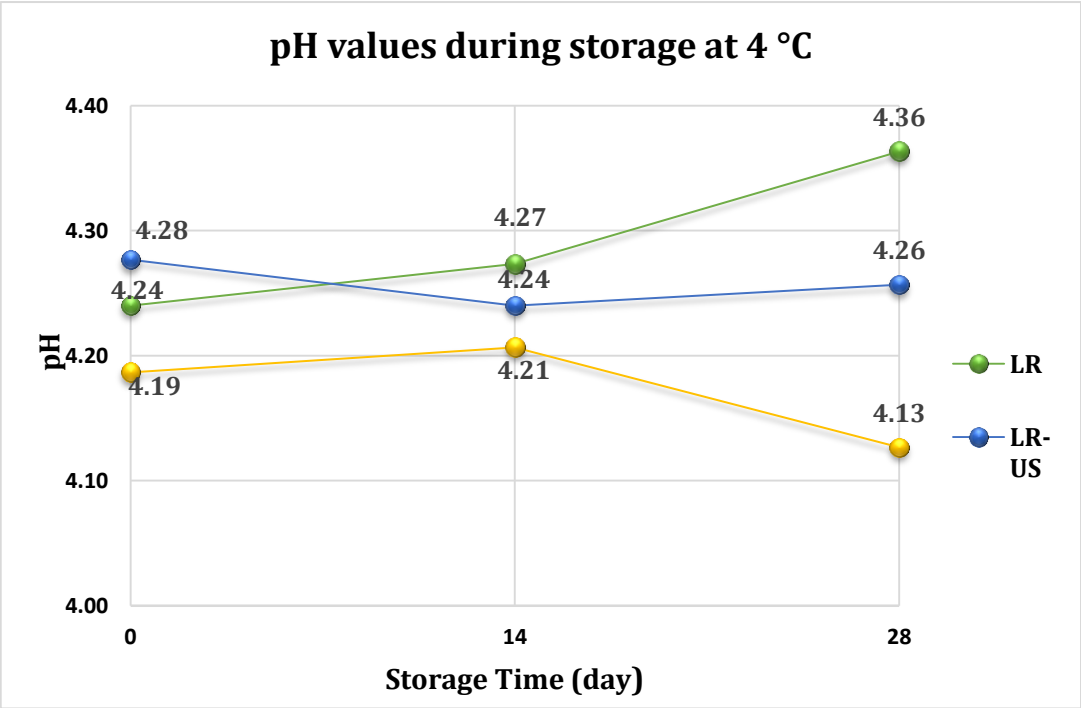

Figure 25: pH values for the three types of probiotic tomato juice (LR, LR-US and LR-US-MC) during storage time (28 days) at 4° C.

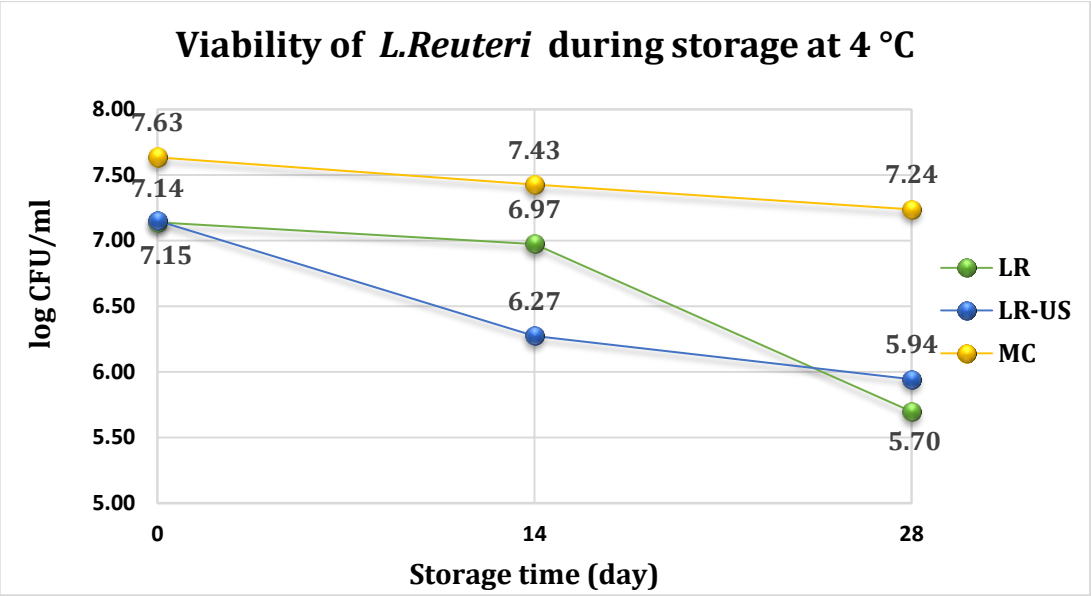

Figure 26: Viability reduction in case of three different probiotic tomato juice during storage time 28 days at 4° C.

The three juices show a different pH trend. For tomato juice added with the probiotic in free form, the pH increases over time, reaching the highest value of 4.36 ± 0.01 at the end of storage. Probably, the microorganism is able to use the nutrients present in the beverage to produce some metabolites that contribute to increase pH (65). However, it should be considered that, although in the first 14 days the vitality remains almost constant, in the last two weeks there is a significant decline($P<0.05$). Therefore, it could probably be argued that the increase in pH is due to both microbial metabolism and a reduction in the microbial load. The juice which, instead, has a more stable pH during storage at 4 ° C is that added with sonicated cell culture. In fact, from t_0 to t_{28} days the pH varies by 0.02. Therefore, compared to the analysis carried out on the fermentative metabolism (section 3.1.1) where, in ideal fermentation conditions (37 °C) and in an ideal medium for growth, the sonication treatment has a temporary effect, the sonication / refrigeration combination is effective for

have a probiotic juice with physical-chemical characteristics stable over time. However, the LR and LR-US samples show a rather similar trend in viability. For both, highly significance reduction is observed during storage($P<0.05$). If for the first the greatest decrease is recorded in the last two weeks, for the second the opposite phenomenon was monitored. The reduction in the microbial load at the end of the storage is of 1,44 log CFU/ml, while for the second it is of 1,21. Minimal differences in vitality are therefore observed. In general, this reduction in viability could be associated with the low pH of the juice.

The tomato juice added with the microcapsules differs from the previous two both in terms of pH and vitality. In this product, the pH decreases during storage (reduction of 0.06). This phenomenon moves away from what was expected. Instead, the results obtained regarding the microbial load confirm those expected. The sodium alginate microcapsule, in fact, minimizes the matrix / probiotic interactions. Therefore, considering the objectives set, that is to have a stable product, and that a portion of tomato juice is 125-200 ml, the best probiotication system, at refrigeration temperatures, is the addition of sonicated culture.

After assessing the results, we can see that the pH value for LR-US-MC tomato juice sample after storage for 28 days at 4 °C was the lowest compared with LR and LR-US tomato juice samples as shown in Figure 25. This result is related to the highest viability of L. reuteri shown in Figure 26, This is one of our targets in this study, but it is necessary to take into

account the second target that is to preserve our juice from any sensory attributes changes.

Table 9: pH-values for three different probiotic tomato juice samples during the storage time at 20^{o} C.

Time (days)	Sample		
	LR	LR-US	LR-US-MC
0	4.24 ± 0.03a,[1]	4.27 ± 0.04a	4.18 ± 0.07a
5	3.61 ± 0.01a	3.71 ± 0.06a	4.07 ± 0.06a
10	3.45 ± 0.03a	3.45 ± 0.02a	4.00 ± 0.04b
15	3.38 ± 0.05a	3.32 ± 0.08a	4.05 ± 0.04b
20	3.29 ± 0.03a	3.36 ± 0.04a	4.02 ± 0.06b
28	3.30 ± 0.01a	3.30 ± 0.03a	3.87 ± 0.05b

[1] The same letters in the same raw indicate that the differences for each storage time and each sample are not significant, (Two-way ANOVA and Bonferroni test for multiple comparisons, P >0.05).

Table 10: The viability of L. reuteri in case of three different probiotic tomato juice samples during the storage at 20^{o} C.

Time (days)	Sample		
	LR	LR-US	LR-US-MC
0	7.14 ± 0.08a,[1]	7.15 ± 0.20a	7.63 ± 0.09b
5	8.70 ± 0.12a	9.14 ± 0.16b	7.26 ± 0.14c
10	9.17 ± 0.10a	9.13 ± 0.03a	7.41 ± 0.03b
15	9.12 ± 0.05a	9.09 ± 0.05a	7.27 ± 0.21c
20	8.70 ± 0.56a	9.00 ± 0.12a	6.80 ± 0.21c
28	8.34 ± 0.17a	8.85 ± 0.08b	7.08 ± 0.23c

[1] The same letters in the same raw indicate that the differences for each storage time and each sample are not significant, (Two-way ANOVA and Bonferroni test for multiple comparisons, P >0.05).

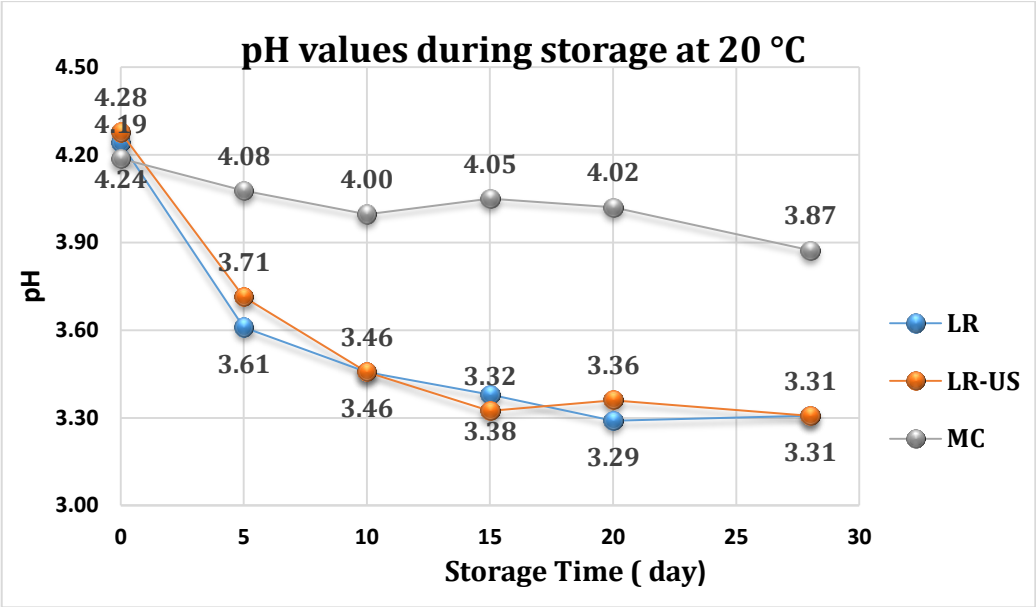

Figure 27: pH values for the three types of probiotic tomato juice (LR, LR-US and LR-US-MC) during storage time at 20° C.

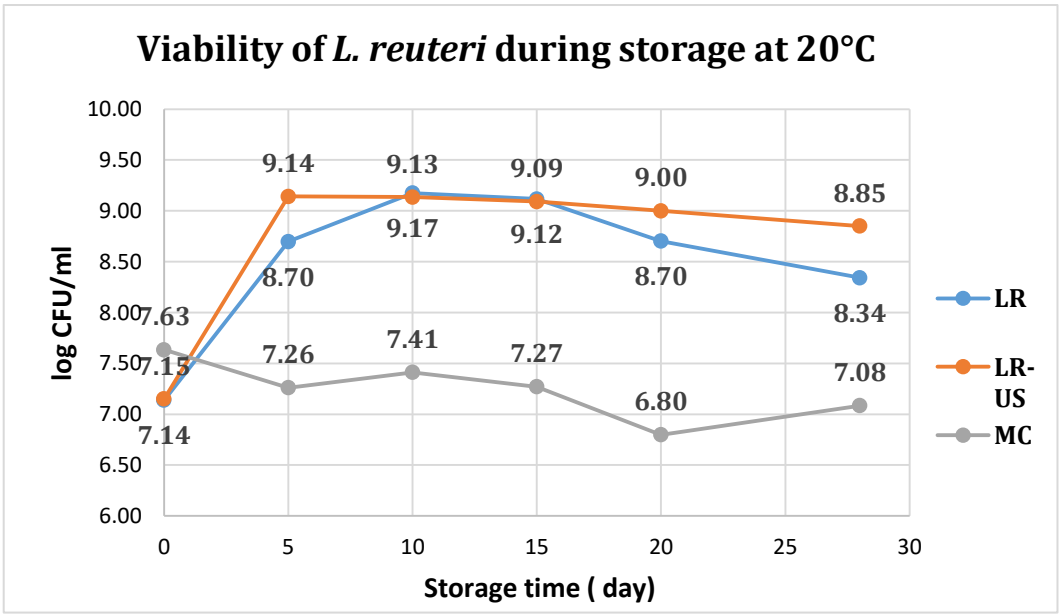

Figure 28: Viability of L. Reuteri in case of three different probiotic tomato juice during storage time at 20°

On the other hand, Table 9 and Table 10 show the results of pH value and viability of L. *reuteri* in case of three samples stored for 28 days at 20 °C, these tests were done from time zero every 5 days.

The three juices show different pH trend (Figure 27). For tomato juice added with probiotic in free form, the pH significantly decreases over time($P<0.05$), reaching the lowest value of 3.30 ± 0.01 at the end of storage. Probably, the microorganism fermentative metabolism is higher at storage temperature (20 °C). So, high production of lactic acid leads to pH reduction. Moreover, the microbial load increases (1.2 log CFU/ml) during the storage (Figure 28). These results agree with each other. In fact, the increase in metabolic activity is associated with proliferation. However, the LR and LR-US samples show a rather similar trend in pH and viability, and the greatest changes for both samples in case of pH value and viability are recorded in the first 5 days of storage($P<0.05$). Unlike LR-US juice stored at 4 ° C, the sonication treatment did not induce any changes, in terms of metabolic activity and viability, on LR-US juice stored at 20 ° C. In this last pH reach the lowest value of 3.30 ± 0.03, and the microbial load increases (1.7 log CFU/ml) at the end of storage. For both samples, LR and LR-US at 20 °C, probably, these results are related to the higher storage temperature closes to the optimal temperature for fermentation and growth (37 °C).

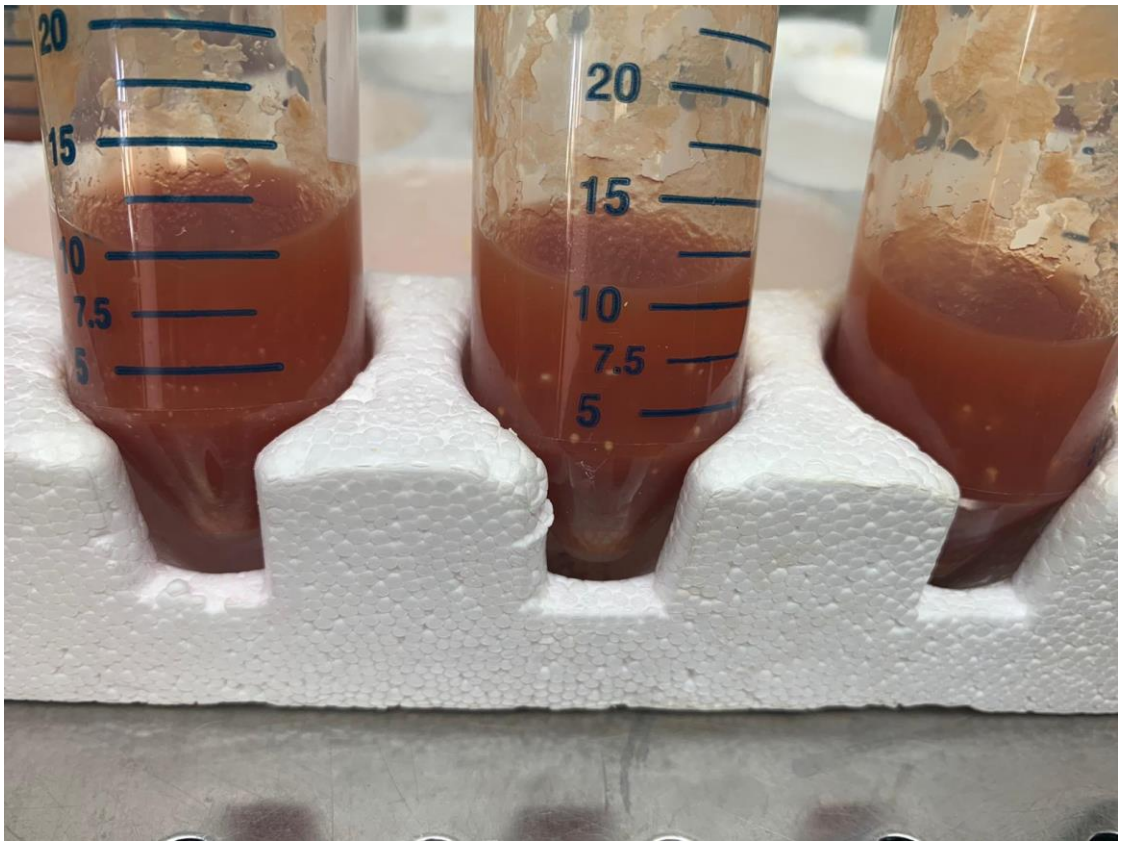

Figure 29: Aggregation of L. reuteri in sonicated samples at 20 °C storage.

During storage at 20 °C, on the surface of the tomato juice added with the sonicated cell suspension appears aggregation. As shown in Figure 29, the cells are present in the form of colonies. Data on bacterial cell aggregation induced by sonication treatment have been reported in the current literature (51).

In addition, we find that the pH value for LR-US-MC tomato juice sample is the highest one compared with other two samples. This value is related to the lowest microbial load for this sample. Sodium alginate microcapsules create a physical barrier between the probiotic and the external environment. Consequently, the supply of nutrients to the probiotic is subject to the permeability of the microcapsule. However, the microcapsule has a structure that guarantees the permeation of the minimum quantities of

nutrients to prevent growth and at the same time keep the cells viable. About our targets; the reduction in pH of LR-US-MC is not too much comparing with pH at t_0 (ΔpH= 0.31), but in other two samples ΔpH = 0.94 for LR, 0.97 for LR-US, the second targets, even if the viability in LR-US-MC is the lowest here but it stills acceptable according to the probiotic foods standards (10^6-10^7 CFU of viable probiotic bacteria / ml or gm of food) (29).

Finally, the results of this study that are combined together from both of storage temperature (4^o and 20° C) confirm that the best storage temperature for *L. reuteri* sonicated and microencapsulated (LR-US-MC) tomato juice is 4°C; the pH (4.13) and viability (7.24 log CFU/ml) value are higher at 4^oC storage temperature than pH (3.87) and viability (7.08 log CFU/ml) value at 20^oC.

3.4 Sensory Acceptance

Table 11: scores of the perceived differences between the reference R and the tomato juice added with the microorganism in free form (LR) and in the form of microcapsules (MC-LR-US) stored at 4 ° C and the hidden control.

Tester	Hidden control	LR	MC-LR-US	Tester	Hidden control	LR	MC-LR-US
1	0.7	10	6.7	16	0.3	4.7	5.7
2	6.7	6.7	6.7	17	0.4	1.7	7.4
3	0.1	9.2	8.9	18	8.0	8.0	0.0
4	0.0	8.1	9.3	19	0.2	0.2	2.4
5	0.0	2.7	1.5	20	0.0	6.7	2.1
6	0.0	8.3	7.1	21	0.0	2.6	1.5
7	0.4	6.9	5.0	22	0.0	5.1	2.8
8	9.8	0.1	5.0	23	2.1	7.8	2.0
9	8.2	8.6	7.8	24	0.3	8.4	9.0
10	0.0	5.4	4.4	25	8.5	7.0	0.1
11	0.9	8.1	10	26	0.0	2.9	5.0
12	0.0	8.7	3.2	27	0.2	5.5	8.3
13	4.1	4.8	5.8	28	0.0	5.0	9.1
14	0.2	5.0	9.8	29	0.1	9.5	7.1
15	0.0	0.9	1.7	30	0.0	10	10

Each taster was asked to rate the perceived differences between the reference and the three samples on a scale from 0 to 10. Table 11 shows the scores of the perceived differences between the reference R and the tomato juice added with the microorganism in free form (LR) and in the form of microcapsules (MC-LR-US) stored at 4 ° C and the hidden control.

On the basis of the collected scores, the difference between the scores obtained for the LR sample with the hidden reference and between the scores of the MC-LR-US sample with the hidden reference was calculated mathematically. In addition, the difference between the two probiotic juices was also evaluated. The arithmetic difference was then analysed through the Dunnett test. 95% confidence interval; there is highly significance

differences between hidden R VS. LR (P<0.05), and significance differences between R VS. MC-LR-US (P<0.05). In contrast, the differences between two probiotic juices LR VS. MC-LR-US are not significant (P>0.05).

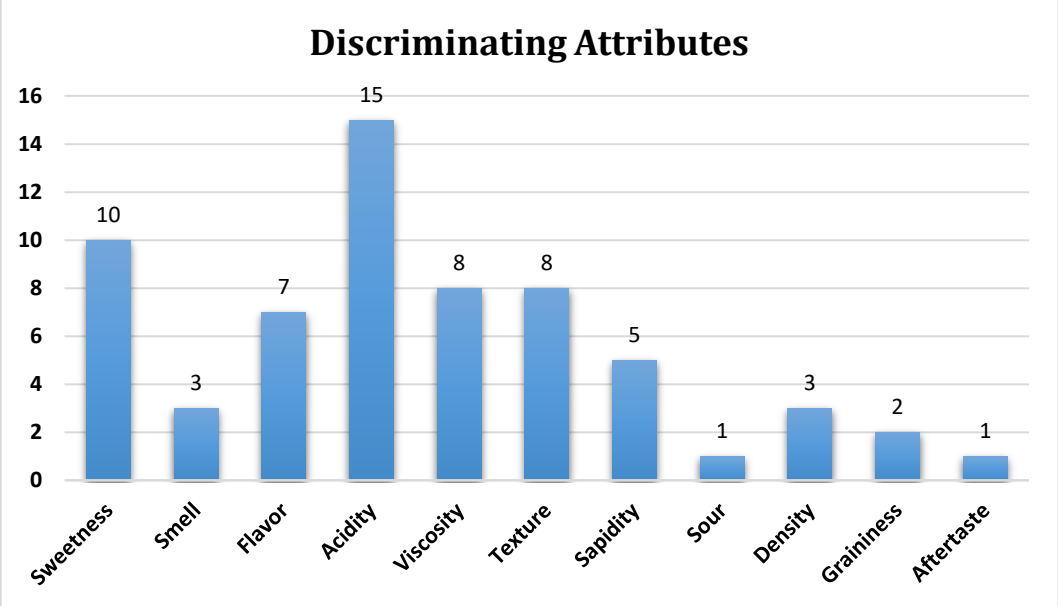

Figure 30: Frequency of many attributes that differentiate the samples from the reference.

During the evaluation phase, the panel of tasters was asked to indicate the attributes that differentiate the samples from the reference. Figure 30 shows the frequency of each attribute.

The three attributes that have the greatest number of citations are acidity (15), sweetness (10) and viscosity and texture (8). In general, however, it is possible to state that probiotic affects gustatory, olfactory and mechanical parameters (density and viscosity).

Chapter Four
Conclusion

This thesis project is part of the innovation and evolution phase concerning the functional food market, in particular probiotic ones. Through our study, the possibility of stabilizing a probiotic tomato juice with attenuation techniques, such as sonication and microencapsulation was evaluated.

Furthermore, with a view to expand the group of probiotic foods, storage at refrigeration temperatures with storage at room temperature was compared. In addition, sensory analysis was not excluding from the evaluation parameters. From the results that obtained from this study, we can say that the best power and duration time combination for sonication treatment is 57 W for 6 min, because the acceptable values of ΔpH and viability for *L.reuteri* were achieved from this combination compared to other combinations.

In addition, we can say that the best probiotication system is sonication followed by microencapsulation of *L. reuteri*, because pH value for sonicated *L. reuteri* microcapsules tomato juice sample was the highest one compared to other samples, the microbial load also was acceptable according to the probiotic food standards (10^6-10^7 CFU of viable probiotic bacteria / ml or gm of food). Moreover, the best storage temperature for probiotic tomato juice was obtained at 4°C for 28 days, because this temperature was led to the minimal chemical-physical compared with the other samples stored at 20^{o}C. Instead, the results of the sensory evaluation

show that the probiotication of tomato juice changes the perception of the product compared to the commercial one. In particular, the differences induced by the addition of the probiotic are perceived on gustatory, olfactory and mechanical attributes. From this preliminary assessment, further sensory tests could be built. First of all, to evaluate which of the samples the taster's preference falls on. So to understand if the perceived differences affect the liking of the drink, positively or negatively. Secondly, it might be interesting to understand how health food information may or may not modify the preference expressed.

From the results of this study, it is concluded that the sonicated *L. reuteri* microcapsules tomato juice could serve as a health beverage for all people categories especially consumers who are allergic to dairy product. And these results obtained from this study depend on the strain, the type of juice and the polymer that used for microencapsulation.

Although, the results obtained show the feasibility of probiotic tomato juice and the use of ultrasound and microencapsulation as an attenuation technique, other aspects need to be investigated. First, to study the survival of the probiotic on passage along the gastrointestinal tract, by virtue of the effects shown by sonication on functional properties (survival at low pH and bile salts). In addition, it could be particularly interesting to apply the same protocol on different strains and fruit juices, in order to evaluate the possibility of defining a method applicable also at industrial level.

Finally, the effect of attenuated probiotic tomato juice on the host health will be the focus of research, by performing vivo studies in order to establish a valuable treatment for many diseases such as; cancer, diabetes mellitus and Cardiovascular diseases.

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تأثير تخفيف النشاط الاستقلابي للبكتيريا النافعة على وظائفية عصير الفاكهة

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الملخص

من الممكن اعتبار الموجات فوق الصوتية وكبسلة البروبيوتيك وسيلتين لتعديل عملية التمثيل الغذائي لبكتيريا البروبيوتيك، وبناءاً على ذلك، من الممكن استخدام تلك الوسائل لتقليل درجة الحموضة في المشروبات التي تحتوي على البروبيوتيك بكتيريا.

من الممكن تخفيف التمثيل الغذائي للبكتيريا وإنتاج الأحماض في المشروبات من خلال دمج تقنية الموجات فوق الصوتية وكبسلة البكتيريا كوسيلة فيزيائية.

كبسلة بكتيريا البروبيوتيك تقنية ممكن تنفيذها من خلال تكنولوجيا الاهتزازات التي تهدف أيضا إلى تدعيم الأغذية في بكتيريا البروبيوتيك لتصنف كأغذية وظيفية، تعتبر الكبسولة التي تحتوي على بكتيريا البروبيوتيك ومضبوطة الفتح تحت ظروف معينة من الأمثلة على الأغذية الوظيفية.

بالنسبة إلى تقنية تثبيط التمثيل الضوئي للبكتيريا، لا يتوفر معلومات كافية عن تأثير هذه التقنية على المظهر العام على بكتيريا البروبيوتيك. لذا: النقاط الرئيسية في هذه الدراسة هي:1) دراسة تأثير الموجات الفوق صوتية والكبسلة على بقاء ونمو بكتيريا *Limosilactobacillus reuteri* (*L. reuteri*) DSM 17938.

وعلى درجة الحموضة أيضا عند حقنها في عصير البندورة خلال فترة تخزينها لمدة 28 يوم (درجة حرارة 4 و20 درجة مئوية)، في وسط حمضي (PH2.5) ,وفي وسط يحتوي على عصارة صفراء بتركيز 0.15 %.

2) دراسة تأثير الموجات فوق الصوتية على بقاء البكتيريا.

3) الحصول على منتج نهائي مقبول لدى المستهلك من ناحية كيميائية وفيزيائية وحسيّة.

يتوفر بيانات لتجارب سابقة تم تطبيق الصوتنة فيها باستخدام ثلاث مستويات للطاقة (57، 64، 78 واط) وفترتين زمنيتين (4،6 دقائق)، حيث تبيّن أنّ أفضل زوج بين الطاقة والمدة الزمنية كان 57 واط لمدة 6 دقائق، تم اختيار هذا الزوج على أنه الافضل بناءا على نتائجه المقبولة من ناحية حموضة المنتج وبقاء البروبيوتيك حيّة في المنتج بعد عملية الصوتنة. علاوة على ذلك، تم دراسة الخصائص الوظيفية للمنتج بعد عملية الصوتنة في وسط حمضي 2.5 حيث أن نسبة بقاء البروبيوتيك حية كانت أعلى قبل تطبيق عملية الصوتنة على البكتيريا، على النقيض من ذلك، تبين أن الانخفاض في نسبة بقاء البروبيوتيك حيّة في وسط المياه الذي يحتوي على العصارة الصفراء بنسبة 0.15% كانت مرتفعة أكثر من النصف في حالة البروبيوتيك الغير معالجة بالصوتنة مقارنة بالبروبيوتيك المعالج.

تدعيم عصير البندورة المتوفر بالأسواق بالبروبيوتيك تم تطبيقة بثلاثة اشكال مختلفة (حقن البروبيوتيك كما هو دون معالجة، حقن البروبيوتيك بعد معالجته بالصوتنة، حقن البروبيوتيك بعد معالجته بالصوتنة وحصره داخل كبسولة).

تم ضبط و مراقبة التغييرات في درجة الحموضة الناجمة عن التمثيل الضوئي للبروبيوتيك وكذلك بقائها حيّة خلال تخزينها لمدة 28 يوم على درجة حرارة 4 و 20 درجة مئوية.

أثبتت هذه الدراسة أنّ أفضل درجة حرارة لتخزين *L. reuteri* DSM 17938 المعالجة بالصوتنة والمحصورة داخل كبسولة هي 4 درجة مئوية ; (4.13) PH, والبقاء 7.24 log CFU/ml وهذه القيم أعلى من قيم التخزين على درجة حرارة 20 مئوية; (3.78)PH, والبقاء 7.08 log CFU/ml .